PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68

A1

(11) International Publication Number: **WO 98/36096**

(43) International Publication Date: 20 August 1998 (20.08.98)

(21) International Application Number: PCT/US98/02750

(22) International Filing Date: 12 February 1998 (12.02.98)

(30) Priority Data:
60/040,306 14 February 1997 (14.02.97) US

(71) Applicant *(for all designated States except US)*: E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).

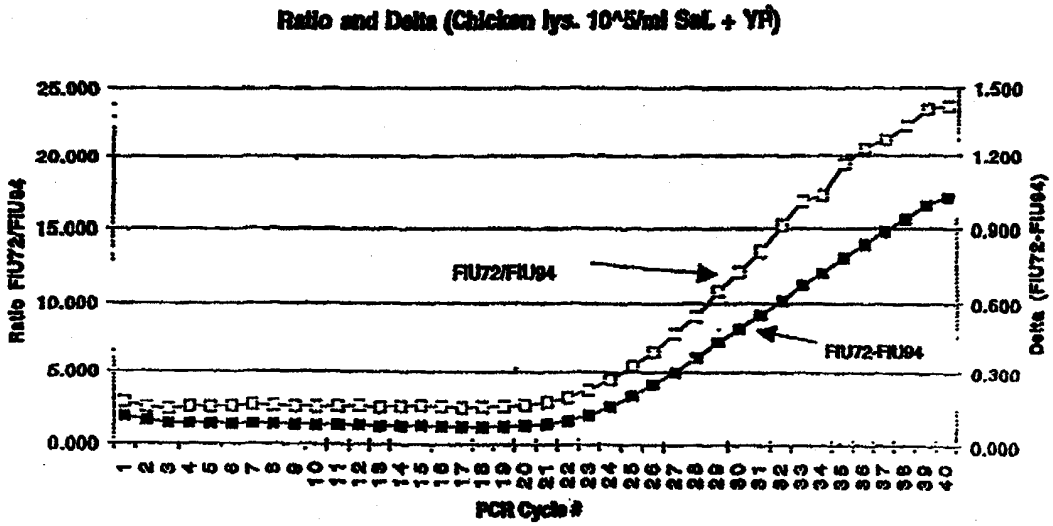
(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BASS, Jay, Kevin [US/US]; 1685 Tulane Drive, Mountain View, CA 94040 (US). JENSEN, Mark, Anton [US/US]; 1176 Fielding Drive, West Chester, PA 19382 (US). TICE, George, Jr. [US/US]; 57 Church Street, Penns Grove, NJ 08069 (US). TSENG, Susan, Yen-Tee [US/US]; 9 Tobin Court, Hockessin, DE 19707 (US).

(74) Agent: MAJARIAN, William, R.; E.I. du Pont de Nemours and Company, Legal Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).

(81) Designated States: AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GE, GW, HU, ID, IL, IS, JP, KG, KP, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, SL, TJ, TM, TR, TT, UA, US, UZ, VN, YU, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DETECTION OF DOUBLE-STRANDED DNA IN A HOMOGENEOUS SOLUTION



(57) Abstract

The present invention provides a method for distinguishing the presence of dsDNA from ssDNA on the basis of the shape of the waveform generated as a sample of DNA is subjected to cycling denaturing and annealing temperature. Specifically, applicants have discovered that the slope generated by measuring the change in the rate of fluorescence during the annealing portion of the cycle may be used to differentiate between dsDNA and ssDNA. The present method is useful in the identification of dsDNA that has been generated from a specific target organism and has particular application to the detection of food borne pathogenic bacteria.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

TITLE
DETECTION OF DOUBLE-STRANDED DNA IN A HOMOGENEOUS SOLUTION

FIELD OF THE INVENTION

This invention relates to the field of methods for nucleic acid detection and is applicable in homogeneous detection of products in assays such as polymerase chain reaction (PCR).

BACKGROUND OF THE INVENTION

There are many methods available to detect the presence of double-stranded DNA (dsDNA) in an optically complex solution. Typically, these involve separation of the products of an assay from the starting matrix. Methods include analytical centrifugation, equilibrium dialysis, melting temperature profiles, and polyacrylamide gel electrophoresis and the like. Such methodology is complex, expensive and time consuming.

Alternative methods which permit identification of dsDNA in a solution or suspension of the initial matrix without any separation are also known. These methods are termed "homogeneous detection" methods. For example, European Patent 0 487 218 teaches a method for detecting a dsDNA target nucleic acid amplification product in a sample by amplifying a region of the nucleic acid by polymerase chain reaction (PCR), contacting or reacting that region with a marker having a detectable property that changes upon contact or reaction and detecting that change. Fluorescent pigments, especially intercalating fluorescent pigments, are taught. In published Canadian Patent Application 2,067,909, Higuchi discloses a DNA binding agent which provides a detectable signal when bound to double-stranded nucleic acid, which signal is distinguishable from the signal when it is unbound. Higuchi employs an intercalating fluorescent dye (ethidium bromide) for DNA labeling and performs all labeling and amplifications on homogeneous DNA samples.

Similarly, Sutherland et al. (U.S. Patent No. 5,563,037) teach the use of a high affinity fluorescent dye which is from the class of unsymmetrical cyanine dyes having at least two positive charges and a binding constant (K_b) within the range of from about $1x10^4$ to about $5x10^5$ ($molar^{-1}$). The cyanine dyes are used in a homogeneous method on homogeneous DNA samples for the detection of target DNA. Characterization and comparison of the fluorescent signal generated from the bound dye leads to differentiation between the dsDNA target amplification product and single-stranded DNA (ssDNA). Differentiation between signals is made on the basis of increase in emission intensity.

Wittwer et al. in (*BioTechniques* 22, 130, 1997) and in PCR publication WO 9746712 disclose a method for monitoring PCR reactions in homogeneous

DNA samples, using the temperature-sensitive DNA intercalating dye SybrGreen I™. The method of Wittwer et al. monitors the progress of the PCR reaction and quantifies the amplification product according to the change in fluorescence of the dye during thermocycling. Wittwer et al. note that amplification product reannealing may be detected and product quantified by determining the rate of product reannealing from the level of fluorescence and the annealing temperature.

The above cited methods are useful for the detection and quantitation of amplified target DNA in a homogeneous sample. However, each suffers from the inability to distinguish between dsDNA amplified target and non-target ssDNA or dsDNA in the same sample. All of the cited methods use purified, homogeneous DNA sample in the stated amplification reactions. Further, none of the methods using temperature-sensitive intercalating agents use the unique differences in the fluorescence waveform generated at DNA-annealing low temperatures to distinguish and quantify target dsDNA.

The problem to be overcome, therefore, is to develop a homogeneous detection method capable of distinguishing dsDNA target amplification product in a complex mixture of nucleic acid sample. Applicants have solved the stated problem through the use of a temperature-sensitive intercalating agent and the analysis of the fluorescence waveform generated when the agent is bound to either dsDNA target amplification product or non-target ssDNA. Applicants have made the unexpected and surprising discovery that the waveform generated by the dye bound to dsDNA target amplification products has certain distinguishing characteristics that make it possible to detect and quantify the target in a non-homogeneous nucleic acid sample. Measurements are taken in real time at a temperature sufficiently high that the fluorescence level of the background has decreased greater proportionately than the fluorescence from the amplified target DNA. The system is amenable to using real-time fluorescence throughout thermal cycling or to a post-thermal cycling high-temperature measurement. Further, the method of the invention is applicable to the detection of dsDNA target regardless of its source. In practice, the dsDNA may be derived by any primer-directed amplification protocol.

The process of the present invention may be used to detect the presence of a wide variety of target nucleic acids. Generally, these include, but are not limited to, DNA derived from microorganisms such as bacteria, yeasts and fungi, as well as viruses, insects, plants, animals and humans. Of particular interest are pathogenic microorganisms known to contaminate food.

SUMMARY OF THE INVENTION

Applicants have provided a method for distinguishing dsDNA from ssDNA comprising:

(a) generating and recording a fluorescence waveform for a sample mixture by subjecting the mixture to at least one cycle of alternating denaturing and annealing temperatures, the mixture comprising:

(i) a solution suspected of containing dsDNA; and

(ii) an intercalating agent, wherein the intercalating agent generates a detectable signal when bound to a nucleic acid and no signal when unbound;

(b) measuring the rate of change in fluorescence over the annealing portion of the cycle to generate a slope, wherein the slope indicates the presence or absence of dsDNA.

The invention is generally applicable to the detection of dsDNA obtained from target bacteria, such as pathogenic bacteria, which may be present in complex solutions containing various debris and food matrixes. Accordingly, the invention further provides a method for detecting the presence of a target dsDNA in a sample where the target dsDNA released from the target bacteria by a process comprising (i) incubating the sample in a preenrichment medium to resuscitate target and non target bacteria; (ii) incubating the resuscitated target and non-target bacteria in a selective medium to enhance the growth of the target bacteria; and(iii) lysing the enhanced cells of step (iii) in the presence of an effective amount of pronase E and RNAse to release target DNA resulting in the formation of a complex sample mixture; and where the target is amplified by primer directed amplification protocols.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE LISTING

Figure 1 is an example of a waveform generated by the binding of YO-PRO-1™ dye to ssDNA at the annealing temperature of 72°C.

Figure 2 is an example of a waveform generate by the binding of YO-PRO-1™ dye to a 500bp dsDNA standard at the annealing temperature of 72°C.

Figure 3 is a plot of fluorescence over time of a 500 bp dsDNA fragment labeled with YO-PRO-1™ and cycled between 72°C and 94°C.

Figure 4 is a plot of fluorescence over time of a processed sample containing target dsDNA labeled with YO-PRO-1™ and cycled between 72°C and 94°C showing the evolution of a fluorescence waveform characteristic of dsDNA.

Figure 5 is a plot of fluorescence over time of a processed sample containing non-target ssDNA labeled with YO-PRO-1™ and cycled between 72°C and 94°C illustrating a fluorescence waveform characteristic of ssDNA.

Figure 6 is a gel electrophoresis run of DNA isolated from food samples either spiked or unspiked with *Salmonella* bacteria.

Figure 7 is a plot of fluorescence over time of a processed sample containing either 6.4 kb (Figure 7a), 9.6 kb (Figure 7b), or 48 kb (Figure 7c) dsDNA labeled with YO-PRO-1™ and cycled between 72°C and 94°C.

Figure 8 is a plot of fluorescence over time of a processed sample containing either 1.0 kb (Figure 8a) or 2.2 kb (Figure 8b) dsDNA labeled with YO-PRO-1™ and cycled between 72°C and 94°C.

Figure 9 is the fluorescence measurements of a forty cycle PCR amplification of DNA from a sample of chicken lysate spiked with 10^5/mL of *S. typhimurium* in the presence of an effective amount of YO-PRO-1™ over a temperature range of 72°C to 94°C.

Figure 10a is a plot of fluorescence over time, measured at 75°C of varying concentrations of a 500bp standard DNA in the presence of SybrGreen™ dye, in the absence of primer-dimer complexes.

Figure 10b is a plot of fluorescence over time, measured at 75°C of varying concentrations of a 500bp standard DNA in the presence of SybrGreen™ dye, in the presence of primer-dimer complexes.

Applicants have provided one sequence listing in conformity with 37 C.F.R. 1.821-1.825 and Appendices A and B ("Requirements for Application Disclosures Containing Nucleotides and/or Amino Acid Sequences") and in conformity with "Rules for the Standard Representation of Nucleotide and Amino Acid Sequences in Patent Applications" and Annexes I and II to the Decision of the President of the EPO, published in Supplement No. 2 to OJ EPO, 12/1992.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for distinguishing the presence of dsDNA from ssDNA on the basis of the shape of the waveform generated as a sample of DNA is subjected to cycling denaturing and annealing temperature. Specifically, Applicants have discovered that the slope generated by measuring the change in the rate of fluorescence during the annealing portion of the cycle may be used to differentiate between dsDNA and ssDNA.

The method has particular application where it is useful to detect the presence of a specific dsDNA target, generated, for example by PCR, where the only dsDNA in the sample will be the desired product. The method is particularly useful for the detection of dsDNA targets from complex mixtures of bacteria and other debris as will be found in environmental and food samples, since the

generation of the correct slope is substantially independent of factors contributing to non-specific fluorescence.

As used herein the following terms may be used for interpretation of the claims and specification.

The term "a homogeneous detection method" will refer to a method of detecting a specific dsDNA target molecule without the need for separating the target from non-specific contaminants.

The term "complex sample mixture" means a sample containing a mixture of dsDNA target amplification product and non-target DNA. A complex sample mixture may also optionally contain elements of food matrices and other contaminating background materials.

"Nucleic acid" refers to a molecule which can be single-stranded or double-stranded, comprising monomers (nucleotides) containing a sugar, phosphate and either a purine or pyrimidine. In bacteria, lower eukaryotes, and in higher animals and plants, "deoxyribonucleic acid" (DNA) refers to the genetic material while "ribonucleic acid" (RNA) is involved in the translation of the information from DNA into proteins.

The abbreviations "ds" and "ss" will refer to double stranded and single stranded respectively, as applied to the strandedness of nucleic acid molecules.

The term "amplification product" refers to specific DNA fragments generated from any primer-directed nucleic acid amplification reaction. The term "target amplification product" or "amplified target" refers to dsDNA that is generated from a specific target DNA to be identified and quantified by means of primer directed amplification. Target amplification products may be produced by any primer directed amplification method. Target amplification products will generally be dsDNA and will be amenable to being bound by intercalating agents. Target nucleic acids will typically be isolated from organisms and pathogens to be identified by the instant method such as bacteria, viruses and other microorganisms.

The term "primer-directed amplification" refers to any of a number of methods known in the art that result in logarithmic amplification of nucleic acid molecules using the recognition of a specific nucleic acid sequence or sequences to initiate an amplification process. Applicants contemplate that amplification may be accomplished by any of several schemes known in this art, including but not limited to the polymerase chain reaction (PCR) or ligase chain reaction (LCR).

The term "nucleic acid replication composition" refers to a composition comprising the ingredients necessary for performing nucleic acid replication. Nucleic acid replication compositions may be provided in a variety of forms including liquid mixtures as well as tableted reagents. If PCR methodology is

selected, the amplification method would include a replication composition consisting of, for example, nucleotide triphosphates, at least one primer with appropriate sequences, DNA or RNA polymerase, and proteins.

The term "target nucleic acid" or "target DNA" refers to the nucleic acid fragment to be detected by the present detection method.

The term "non-target nucleic acid" or "non-target DNA" will refer to any nucleic acid other than the target DNA. Non-target DNA may be either single-stranded or double-stranded, may be an amplified product or genomic, but will not be unique to the organism or pathogen from which the target DNA is obtained.

The term "target microorganism" or "target organism" or "target bacteria" will refer to a microorganism to be isolated and identified by the present method. Target microorganisms may be either prokaryotic, or eukaryotic and may be members of defined mixed cultures, or exist as contaminants in complex matrices. Target microorganisms of particular interest are food-borne bacteria.

The term "non-target microorganism or bacteria" or "background microorganism or bacteria" will refer to any organism that is found in the presence of the target organism but is not the target organism. Like target organisms, background organisms may be either prokaryotic or eukaryotic, and may or may not be related genetically or biochemically to the target organism. Those background organisms of most interest in the context of the present application are food-borne bacteria.

The term "selective growth media" will refer to either a solid or liquid growth media specifically formulated to enhance the growth of the target organism and discourage the growth of background organisms. Additionally, selective growth media may contain components designed to facilitate the detection of target organisms, generally by visual inspection.

The term "pre-enrichment media" will refer to either a liquid or solid media designed to encourage the growth of both target and background microorganisms. Microorganisms are inoculated and incubated in pre-enrichment media to enhance the numbers of all microorganisms present and to resuscitate any target organism that may have been damaged in initial sample processing. The pre-enrichment media of the present invention is buffered to allow for the variations in pH of a variety of different food matrices.

The term "plasmid" or "vector" used herein refers to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules. Plasmids generated in the course the present invention are used principally as reagents in the primer-directed amplification protocol.

The term "primer" refers to a nucleic acid fragment or sequence that is complementary to at least one section along a strand of the sample nucleic acid, wherein the purpose of the primer is to sponsor and direct nucleic acid replication of a portion of the sample nucleic acid along that strand. Primers can be designed to be complementary to specific segments of a targeted sequence. In PCR, for example, each primer is used in combination with another primer forming a "primer set" or "primer pair"; this pair flanks the targeted sequence to be amplified. The term "primer", as such, is used generally by Applicants to encompass any sequence-binding oligonucleotide which functions to initiate the nucleic acid replication process.

The term "intercalating agent" means a temperature-sensitive fluorescent agent capable of intercalating into nucleic acid molecules. The term "intercalating agent" will be used interchangeably with the term "dye". Intercalating agents emit a fluorescent signal when intercalated into the nucleic acid and will not generate any signal when not intercalated. Typical of intercalating agents are the cyanine dyes such as YO-PRO-1™, or SybrGreen™ available from Molecular Probes, Inc. (Eugene, OR, USA).

The term "nucleic acid standard" refers to a nucleic acid fragment of similar size and base composition to the target DNA where the standard is used to generate a calibrating waveform for comparison to the waveform generated by dye binding to dsDNA target.

The term "waveform" or "fluorescence waveform" means the characteristic wave pattern generated by an intercalating agent when bound to a nucleic acid molecule measured at a specific temperature or temperature range over a specific period of time. The waveform is not to be limited to any minimum portion of the wave generated, provided that the portion of the wave is characteristic of the nucleic acid to which the intercalating agent is bound. Fluorescence waveforms may be specific or non-specific depending on whether the intercalating agent is bound to target or non-target nucleic acids.

The term "annealing temperature" will refer to the temperature at which a specific intercalating agent, when bound to ds target DNA amplification products, generates a waveform having a slope that is distinguishable from the slope of the waveform when the agent is bound to non-target, ssDNA. Where the intercalating agent is a member of the cyanine dyes, annealing temperatures are in the range of about 65°C to 90°C.

The term "denaturing temperature" refers to the temperature at which nucleic acid molecules typically denature and corresponds to the temperature at which intercalating agents, suitable in the present invention, show a decreased fluorescence when bound to DNA.

The term "tolerance" as applied to annealing temperature will refer to the degree of variation permitted around the annealing temperature.

The term "Fluorescent Intensity Units" will be abbreviated "FIU".

The term "TDF" is an acronym meaning temperature dependent fluorescence and refers to the method of the present invention where the presence of dsDNA target amplification products are detected in a complex mixture on the basis of a specific fluorescence waveform generated at a specific annealing temperature by the binding of an intercalating agent to the amplified target DNA.

"Standard BAX™ sample preparation" refers to a protocol for the processing of samples containing food and target organisms involving selective enrichment of the target organism, and treatment of the cell lysate with pronase E. The standard BAX™ method of sample preparation is fully described in PCT US96/15085 and in the examples.

"Modified BAX™ sample preparation" refers to a modified BAX™ sample preparation protocol for the processing of samples containing food and target organisms where the modifications include additional pronase E treatment and a treatment with RNAse.

"BAM" means the FDA Bacteriological Analytical Manual published and distributed by the Association of Analytical Chemists, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301.

The present invention is a homogeneous detection method, referred to as the temperature dependent fluorescence (TDF) method for the detection and quantitation of dsDNA target amplification products in a complex DNA sample mixture. Double-stranded amplified target DNA is generated from an organism and contacted with a temperature-sensitive intercalating agent. The intercalating agent emits a unique fluorescence waveform when bound to the double stranded amplified product as opposed to when bound to single stranded non-target DNA. The method has particular application for the identification of double stranded target DNA representative of pathogenic bacteria known to contaminate food.

The invention capitalizes on the observation that real-time fluorescent signal of amplified target dsDNA fragments exhibit an increasing fluorescent signal with time when the temperature is decreased from a DNA denaturing temperature to a DNA annealing temperature. This signal is characterized by a rising-sloped curve as reannealing of two complementary single strands of DNA occurs. Signal produced by dye-bound mixtures of DNA such as from genomic DNA, as well as signals from background food matrices, exhibit relatively flat-topped wave-forms (see, for example, Figure 1). Resolution of the fluorescent signals on the basis of waveform shape permits the elimination of false positives

that would otherwise occur if mere fluorescence intensity was used as a measure of a probative signal.

The invention further provides a sample preparation process that involves (i) initial incubation in a growth medium, (ii) dilution in growth medium, (iii) further incubation, (iv) lysing to release DNA, and (v) processing in the presence of an effective amount of RNAse. This embodiment is particularly useful when analyzing samples contaminated with food matrices and large amounts of genomic DNA derived from contaminating background, non-target organisms in the sample. Sample preparation reduces the amount of non-target DNA and eliminates the possibility of a false positive in the assay.

Typically, food suspected of containing a pathogenic bacteria is subjected to a target enrichment process which enhances the relative numbers of target organisms (typically food borne pathogens) with respect to background bacteria. Following target enrichment, DNA is amplified using primers designed to amplify known identifying regions of the target organism genome. At some point during the amplification process the intercalating agent is added to the reaction and target identified by fluorescence measurements.

PREPARATION OF TARGET dsDNA

Target dsDNA may be from any source where it is of value to discern the presence of a double-stranded nucleic acid fragment that is unique to a particular organism or is defining for a specific genetic trait. Within the context of the present invention, target dsDNA is amplified to produce target amplification products from pathogenic bacterial organisms known to contaminate food. Of particular interest will be DNA isolated from such organisms *Listeria*, *Salmonella*, and *E. coli*.

Although amplified target dsDNA may be generated by any method, primer directed amplifications are preferred because they have the ability to amplify specific regions of a genome in the presence of high quantities of non-specific DNA.

Sample Preparation

Generally the organism from which the amplified target is to be generated is in very low concentrations in a food sample and is in the presence of high concentrations of food matrix and other background bacterial flora. It is preferable if the target DNA is enriched via a sample preparation process before an attempt is made to amplify and isolate the amplification products.

Generally a food sample is first homogenized into non-selective, liquid medium (pre-enrichment broth), using a stomacher or blender. Food samples contain a diverse spectrum of bacteria, not all of which represent the target. These non-target flora are referred to as background microorganisms or non-target

microorganisms. Often the target microorganisms contained in the food sample are injured in the food processing environment and require incubation in a non-selective broth for resuscitation. A typical pre-enrichment medium for *Salmonella* is lactose broth with slight variations for different foods as described in the Bacteriological Analytical Manual. 6th Edition, Association of Official Analytical Chemists, Arlington, VA (1984).

Following pre-enrichment the sample is incubated in a media which promotes selective growth of the target microorganism. This step allows the sample to be further enriched in growth-promoting medium which may additionally contain selectively inhibitory reagents. A continued increase of target microorganisms is achieved while simultaneously restricting proliferation of background microorganisms. So for example, in the present method, food samples are stomached in the presence of a lactose or similar broth and the pH of the blended mixture is adjusted to between 5 and 10 and nutrients designed to enhance the growth of the target organism are added. The mixture is incubated 24 to 48 hr. Cells are lysed, and subjected to treatment with Pronase E. This procedure is termed the standard BAX™ sample preparation method and is fully described in PCT US96/15085.

Optionally, a modified sample preparation may be performed to reduce the background generated in the fluorescent assay method. It is noted that certain samples exhibit fluorescent waveforms that are satisfactory in configuration but have a relatively low signal level. It is surmised that this results from interference or inhibition from the sample. To reduce or eliminate the background noise and interference from the detection of amplified products, a modified BAX™ sample preparation may be performed. This modified BAX™ method employs additional amounts of pronase E and incorporates an RNAse treatment step. Once the target DNA has been processed in this fashion, primer directed amplification may be performed using primers designed specifically to produce unique and identifying amplification products.

INTERCALATING AGENTS

The present method uses an intercalating agent capable of binding to dsDNA and emitting a fluorescent signal distinguishable from the signal generated when unbound or bound to single stranded DNA. A variety of intercalating agents are known in the art such as propidium iodide (PI) and ethidium bromide (EB) [Sailer et al., *Cytometry* (1996), 25(2), 164-172] Oxazole Yellow [EP 714986], TOTO (1,1'-(4,4,7,7-tetramethyl-4,7-diazaundecamethylene)-bis-4-[3-methyl-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene]-quinolinium tetraiodide), a homodimer of thiazole orange [Axton et al., *Mol. Cell. Probes* (1994), 8(3), 245-50] oxazole orange (YOYO)[Srinivasan

et al., *Appl. Theor. Electrophor.* (1993), 3(5), 235-9] as well as the cyanine dyes [U.S. Patent No. 5,563,037]. Preferred in the present method are the unsymmetrical cyanine dyes such as are discussed in U.S. Patent No. 5,563,037; U.S. Patent No. 5,534,416; U.S. Patent No. 5,321,130 and U.S. Patent No. 5,436,134 hereby incorporated by reference.

Cyanine dyes are particularly suited for use in the present method since they possess binding constants for DNA low enough not to inhibit amplification by most primer directed amplification methods but high enough to still give a detectable signal. Preferred binding constants for the cyanine dyes useful in the present invention are from about from about $1x10^4$ to about $5x10^5$ ($molar^{-1}$).

The preferred dyes are temperature-sensitive in that the fluorescent signal decreases to a lesser degree when the dye is bound to dsDNA target amplification products then when unbound or bound to non-target ssDNA. Suitable intercalating agents emit useful fluorescence between the ranges of about 65°C to about 100°C. Fluorescence intensity declines at higher temperatures where the maximum high fluorescing, or denaturing temperatures range from about 94°C to about 100°C. Fluorescence of the intercalating agents increases as temperature decreases where useful annealing fluorescing temperatures are in a range of about 65°C to about 90°C and where temperature of about 70°C to about 85°C are preferred.

Particularly suitable in the present invention are the cyanine dyes YO-PRO-1™ {Quinolinium, 4-[(3-methyl-2(3H) benzoxazolylidene)methyl]-1-[3 (trimethylammonio)propyl]-, diiodide} and SybrGreen™, available from Molecular Probes, Inc. (Eugene, OR, USA). These dyes are particularly suited for use in the present invention due to their high extinction coefficient, near zero fluorescence when unbound, suitable binding affinity to double-stranded DNA and reasonable photostability. These dyes are sufficiently resistant to the elevated processing temperatures at the time intervals used to provide an effective signal in use.

WAVEFORM GENERATION

Target amplification

Applicants contemplate that amplification may be accomplished by any of several schemes known in this art, including but not limited to the polymerase chain reaction (PCR), ligase chain reaction (LCR) or strand displacement amplification (SDA). If PCR methodology is selected, the replication composition would include for example, nucleotide triphosphates, at least one primer with appropriate sequences, DNA or RNA polymerase and proteins. These reagents and details describing procedures for their use in amplifying nucleic acids are provided in U.S. Patent No. 4,683,202 (1987, Mullis, et al.) and U.S.

Patent No. 4,683,195 (1986, Mullis, et al.). If LCR methodology is selected, then the nucleic acid replication compositions would comprise, for example, a thermostable ligase, e.g., *T. aquaticus* ligase, two sets of adjacent oligonucleotides wherein one member of each set is complementary to each of the target strands, Tris HCl buffer, KCl, EDTA, NAD, dithiothreitol and salmon sperm DNA. (See, for example, Tabor, S. and Richardson, C. C. (1985) *Proc. Acad. Sci.* USA 82, 1074-1078).

If the SDA methodology is employed, amplification may be accomplished using either one or two short primers containing a site for *HincII* digestion, an exonuclease deficient DNA polymerase, *HincII* restriction enzyme and the bases dGTP, dCTP, dTTP and deoxyadenosine 5'[a-thio]triphosphate (dATP[aS]). The SDA protocol including the necessary materials is outlined in Walker, et al., *Proc. Natl. Acad. Sci. U.S.A.*, 89, 392, (1992).

Within the context of the present method, a sample suspected of containing target organisms is subjected to a sample preparation protocol and DNA is isolated. Isolated DNA is then amplified according to a standard method for primer directed amplification. Typically, PCR is used and follows a standard thermocycling procedure in the presence of an appropriate nucleic acid replication composition. A suitable nucleic acid replication composition will contain for example, dATP,dCTP,dGTP, dTTP, target specific primers and a suitable polymerase. Primers will be selected to specifically amplify target DNA. For example, where it is desired to identify the presence of *Salmonella, Salmonella* specific primers P35 and P761 identified as SEQ ID NO:1 and 2 respectively, may be used. If nucleic acid composition is in liquid form, suitable buffers known in the art are used. (Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989)). If the composition is contained in a tabletted reagent, then typical tabletting reagents are included as described in PCT US96/15085. The PCR reagents may also contain a suitable intercalating agent such as YO-PRO-1™ or SybrGreen™ at a final concentration of about 2 uM

The TDF method is dependent on the thermal cycling of reagents, including target amplification products, through annealing and denaturing temperatures while in the presence of effective concentrations of dye. Thermocycling proceeds according to typical cycling times and temperatures. Preferred is a two-temperature PCR protocol cycle of 94°C for 15 sec and 72°C for 180 sec where thirty-five to forty-five cycles are run on each sample. During amplification, it is often the case that the initial cycles of the amplification do not produce amplification products at concentrations high enough to be detected on the basis of waveform analysis. Since these products will generally be detected

only in the later cycles of the amplification reaction, the initial thermal cycling is accomplished using commercially available thermal cycling units, such as the Perkin Elmer 9600. After initial thermal cycling, the reagents are transferred to the TDF apparatus (described below) for the final cycles where the apparatus is equipped with the components necessary for fluorescence waveform detection and analysis.

The TDF apparatus is comprised of a temperature-controlled aluminum platen having wells to receive reaction vessels such as clear plastic tubes or wells for holding the PCR reaction reagents. During nucleic acid amplification, fluorescence is continuously measured by a fluorometer provided with two fiber optic bundles for localized excitation and detection of emission. Fluorescence waveform generation is monitored and analyzed by a data acquisition system incorporating a computer and software created for the analysis.

The character of the waveform generated is a function of temperature, concentration of the dye, the size of the DNA, the DNA binding constant of the dye and the presence of contaminating species in the reaction melieu. The dye may be added at the beginning of a PCR reaction and used to monitor the progress of the reaction, measuring fluorescent output at each cycle. The generation of the distinguishing waveform during the thermocycling signals the production of the desired dsDNA amplification product.

Although it is convenient to incorporate the dye into the amplification procedure, it should be noted that the present method is capable of detecting the dsDNA simply during a procedure that cycles the temperature between a denaturing and annealing fluorescing temperature, independent of any PCR reaction.

Denaturing fluorescing temperatures may vary from about 94°C to about 100°C where a temperature of about 94°C is preferred. Annealing fluorescing temperatures may vary from about 65°C to about 90°C where a temperature range of about 70°C to about 85°C is preferred.

Characteristic Fluorescence Waveform

A key distinguishing characteristic of the present method of analysis is the slope of the waveform generated by the binding of the dye to amplified target dsDNA as the reaction cycles through the annealing temperature.

The slope of the waveform generated by dye binding to dsDNA is clearly distinguishable from that generated by binding to ssDNA alone. For example, Figure 1 isolates the waveform slope for ssDNA labeled with dye at an annealing temperature of 72°C. In contrast, Figure 2 illustrates the slope of the waveform generated by the binding of dye to dsDNA amplified target at the same temperature.

As has been noted, the ability to generate a distinguishing waveform using the preferred intercalating agents is dependent on certain temperature parameters. The dyes of the present method are used in a temperature cycling regime where the temperature fluctuates between about 65°C and 100°C. The part of the waveform generated during this temperature cycle that is most characteristic of the nature of the bound DNA is the part generated during the annealing temperature, generally at about 72°C to about 85°C.

Characterization of the fluorescence waveform is, in some measure, dependent on the extent of temperature fluctuation in the annealing fluorescing temperature portion of the cycle. Dramatic fluctuations in temperature in this part of the cycle will cause perturbations in the slope of the waveform so that identification of dsDNA target will be difficult. Temperature fluctuations of no more than 0.5°C over the course of the annealing cycle will give a suitable waveform slope, where a fluctuation of no more than 0.1°C is preferred. [Annealing cycles are typically from about 15 seconds to 10 minutes, where a range of 15 seconds to 7 minutes is preferred, and a range of 15 sec to about 3 minutes is most preferred.] Thus, a suitable waveform will be generated where the annealing temperature is fixed at a temperature between 65°C and 100°C and where the fixed temperature has a tolerance of no more than about 0.1°C to about 0.5°C. So, for example, where the annealing temperature is fixed at 72°C with a tolerance of 0.1°C, the annealing temperature must not fluctuate any higher than 72.01°C and no lower than 71.09°C during the annealing portion of the cycle.

Where the sample containing the target DNA is contaminated with a significant amount of non-target ssDNA the opportunity for primer-dimer generation is high during amplification. When bound to dye, primer-dimer constructs will generate a waveform similar to that seen for dsDNA and will increase the possibility of false positives in the method. In these situations it is preferable if the annealing temperature is raised to between about 75°C to about 85°C where about 80°C is preferred. While not meaning to suggest a rationale, it is thought that at this elevated temperature the primer-dimer melts thereby reducing the amount of primer-dimer fragments that are available to bind the intercalating agent and thus reducing signal interference with the target nucleic acid base pair fragment.

There are a variety of methods for distinguishing a fluorescence waveform indicative of dye binding to dsDNA a waveform for ssDNA. Some approaches include correlation coefficients, curve-fitting, and parameter extraction such as slope, delta, and ratio as used in Example 2. Applicants prefer the use of the extracted parameter slope because of its simplicity of calculation and accuracy.

As noted above measurement slope of the relevant portion of the waveform is made during the annealing portion of the thermocylcing process. Measurement of absolute fluorescence is not sufficient to make a distinction between dsDNA and ssDNA. However, measuring the rate of change in fluorescence at one minute after the change to 72°C (dF/dt @ 1') does provide some differentiation between the homogeneous dsDNA and ssDNA. This difference can be amplified by normalizing the dF/dt @ 1' based on the absolute fluorescence at about one minute after the temperature decrease.

When this analysis was applied to specific pathogenic bacteria a range of threshold slope values were determined that were distinctive of each organism. By analyzing the slopes generated over this series of bacteria it was determined that threshold slope values of greater than about 0.09 were indicative of dye binding to dsDNA, where values lower than about 0.09 were characteristic of dye binding to ssDNA.

Effect Of Dye DNA Binding Constant

Generation of a specific fluorescence waveforms, indicative of dye binding to dsDNA are affected by dye concentration and the binding constant of the dye chosen. The binding constant of YO-PRO-1™ is 1.5 E5/M (from DNA partition coefficient in 10% ethanol/water solution, see Molecular Probes, HANDBOOK OF FLUORESCENT PROBES AND RESEARCH CHEMICALS, by Richard P. Haugland, 1992-1994, pp. 223-224) and does not significantly inhibit PCR. Dyes with binding constants that vary significantly from this figure are not suitable in the present invention. For example, TOTO-1 (Molecular Probes, Eugene OR), has a binding constant two orders of magnitude greater at 2.0 E7/M but generates no PCR product in similar tests. Suitable binding constants for the intercalating agents useful in the present invention are from about from about $1x10^4$ to about $5x10^5$ ($molar^{-1}$), in which are included both the dyes YO-PRO-1™ and SybrGreen™.

Dye concentration will have an effect on binding and subsequent waveform generation. Saturation of sample subjected to amplification with dye will inhibit the reaction and no waveform will be generated. Appropriate concentration is readily determined from vendor's material and/or simple experimentation. For the purposes of the present invention dye concentrations of from about 0.1 uM to about 20 uM are suitable where concentrations of about 1.0 uM to about 6 uM are preferred.

Effect Of Sample Contamination

In field samples where the present method is used to detect food born pathogens a high level of contamination is seen. The source of the contamination is generally from both food matrices and high levels of non-target bacteria. High

levels of contamination will have the effect of increasing the background fluorescence and obscuring the waveform patterns so that the presence of amplified target will be masked. These effects are illustrated in Table II and in Example 5. To eliminate some of the contamination a sample preparation protocol is employed. The protocol modifies the standard BAX sample preparation method by including higher concentrations of pronase E and an RNAse. These modifications result in decreased background fluorescence as can be seen from Table II, in Example 5.

Effect Of DNA Fragment Size

The size of the nucleic acid fragment to be detected will affect the character of the waveform generated. Waveforms were analyzed on DNA fragments ranging from 500 bp to 48,000 bp. Under optimal conditions of temperature and dye concentration it was observed that a waveform, characteristic of typical target amplification products, occurred between 500 bp and 10,000 bp where fragments of lengths greater than 10,000 bp gave waveforms that were difficult to distinguish from non-target DNA.

While not meaning to suggest a rationale, Applicants surmise that the mechanism behind the observed transition at 10,000 bp is most likely a function of the combined effects of both re-annealing of the complimentary DNA strands as well as the intercalation of the fluorescent dye. It is thought, for example, when short denaturing times on the order of 15 seconds are used, the larger DNA fragments reanneal more quickly than the smaller fragments because the denatured strands do not have sufficient time to diffuse away from each other, thereby allowing the intercalating fluorescent dye to bind faster. This results in a very sharp rise in the signal following the change from the high denaturing temperature to the low annealing temperature producing the observed squarewave, (see Figure 1, for example). In contrast, it is thought that since in smaller DNA fragments the denatured strands can diffuse further away from each other, these fragments appear to reanneal more slowly and signal generation continues past the temperature transition point resulting in a sawtooth-shaped waveform. It is contemplated that the dye concentration and the dye DNA binding constant also affect the curve shape for any size DNA fragment.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The analysis related above will permit the detection and quantitation of dsDNA targets in complex sample mixtures within the parameters described.

To determine the manner and characteristics of the waveform generated by the binding of an intercalating agent to dsDNA, a 500 bp dsDNA fragment was labeled and cycled between 72°C and 94°C and fluorescence was plotted against

temperature, as described in Figure 3. From this analysis a typical waveform pattern was determined.

To determine the utility of the TDF system in samples containing food matrices, food samples were processed either alone or spiked with known numbers of *Salmonella* bacteria. The complex mixtures were subjected to PCR in the presence of the intercalating dye YO-PRO-1™ and analyzed both by gel electrophoresis and by monitoring fluorescence of the life of the PCR thermocycling. Figure 4 illustrates the fluorescence monitoring of the PCR cycle of the spiked sample. The presence of target is confirmed by the fluorescence waveform indicative of dye binding to dsDNA, and having a distinctive slope at and annealing temperature of 72°C. The presence of target in these samples is confirmed by the gel electrophoresis seen in Figure 6. In contrast, fluorescent monitoring and gel electrophoresis of unspiked samples analyzed in an identical fashion containing only food matrix, confirmed the lack of target. Figure 5 shows the monitoring of the unspiked samples, and illustrates the characteristic fluorescence waveform generated by dye binding to non-target, ssDNA. The absence of target in these samples was confirmed by gel electrophoresis as shown in Figure 6.

The TDF method recognizes the fact that the fluorescence waveform is a function of the size of the labeled DNA fragment. In general, the shorter the fragment the more dramatic the waveform slope in the annealing fluorescing temperate range. In contrast, fluorescence waveforms generated from large fragments give slopes approximating those given by non-target, ssDNA. This effect is clearly seen in Figures 7 and 8 where samples of DNA ranging from 1.0 kb to 48 kb were reacted with dye and fluorescence was monitored over a temperature range of 72°C to 94°C. The slopes of the 6.4 kb, 9.6 kb and 49 kb fragments were considerably flatter (Figure 7) than those generated by the 1.0 kb and 2.2 kb fragments (Figure 8).

The effect of subjecting the bacteria contaminated food matrices to a modified BAX™ sample preparation demonstrated that a cleaner complex sample mixture enhanced the fluorescent signal. Food samples containing certain concentrations of *Salmonella* were subjected to the standard BAX™ sample preparation protocol and the modified protocol and DNA was extracted and amplified in the presence of the intercalating dye YO-PRO-1™. Comparison of the fluorescence waveforms generated by target DNA subjected to the standard protocol and the modified procedure demonstrated that the modified protocol resulted in an enhanced fluorescent signal (Table II).

In a preferred embodiment the invention may be practiced so as to reduce the fluorescence associated with primer-dimer formation. It was demonstrated,

for example, that where primer-dimers were intentionally introduced into the DNA amplification reaction mixture containing dsDNA target and the waveform generated at an annealing temperature of 72°C that significant signal interference was seen (Example 6, Figure 10a). The primer-dimer signal interference was significantly reduced using a higher annealing temperature (Example 6, Figure 10b).

EXAMPLES

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

GENERAL METHODS

Procedures for plasmid construction and manipulation as well as primer directed amplification are well known in the art. Techniques suitable for use in the following examples may be found in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989).

Materials and methods suitable for the maintenance and growth of bacterial cultures are well known in the art. Techniques suitable for use in the following examples may be found in Manual of Methods for General Bacteriology (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds), American Society for Microbiology, Washington, DC. (1994) or Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA. or Bacteriological Analytical Manual. 6th Edition, Association of Official Analytical Chemists, Arlington, VA (1984).

All reagents and materials used for the growth and maintenance of bacterial cells were obtained from Aldrich Chemicals (Milwaukee, WI), DIFCO Laboratories (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), or Sigma Chemical Company (St. Louis, MO) unless otherwise specified.

The standard BAX™ grow back and lysis procedure is fully described in PCT US96/15085.

The cyanine intercalating agents YO-PRO-1™ and SybrGreen™ were obtained from Molecular Probes (Eugene, OR).

Salmonella specific PCR tableting reagents used in the following examples were obtained commercially from Qualicon part no. 17410530 included in the *Salmonella* screening kit catalog number 17720519.

Listeria spp. tablets used in the following examples were obtained commercially from Qualicon part no 1740538.

Ground turkey, chicken, beef and oregano used in the following examples were obtained from commercial vendors.

The meaning of abbreviations is as follows: "h" means hour(s), "min" means minute(s), "sec" means second(s), "d" means day(s), "mL" means milliliters, "L" means liters.

TDF SYSTEM

The TDF system was assembled from the following principal components: Ericomp Single Block Cycler (Modelxx, California), fluorometer module, and Data Acquisition System. A fluorometer module consisted of 1/16SEX/EM fiber optic bundles (Dolan Jenner), HC124-06 Photomultiplier assembly (Hammamatsu), Halogen Lamp (L523, Gilway), and EX/EM 485/530 filters (Corion). The Data Acquisition system was controlled by Labview software (National Instruments) and included a Macintosh IIvx, NBMIO-16 A/D card (National Instruments), and 5B series I/O and temperature input modules (Analog Devices). An aluminum carrier was machined to hold two thin walled thermal cycling tubes (Perkin Elmer). The carrier had a flat bottom and served as a thermal interface between the Ericomp cycler and the sample tubes. In addition, a Delrin holder was machined to hold the aluminum carrier and to align one sample tube with the EX/EM fiber optic bundles. The other tube was utilized to monitor sample temperature. It contained a thermocouple junction (1TC-40-TT-36-K-3, Technical Industrial Products) embedded in 50 uL of thermal epoxy (Omega 101).

Cell Strains

The following strains were used. *Salmonella typhimurium* having internal designation((D)1084) and *Salmonella enteritidis* having internal designation ((D)706).

Nucleic Acid Amplification Reagents

PCR reagents use in the following examples were in tabletted form and contained effective concentrations of dATP, dCTP, dGTP, dTTP, primer 35 [SEQ ID NO:2] primer 761 [SEQ ID NO:1] and AmpliTaq™ DNA polymerase. The relevant PCR tabletted reagent methodology is fully discussed in PCT US96/15085 hereby incorporated by reference.

Calculation For The Presence Of Amplified Product

In order to determine the possible existence of dsDNA amplified target DNA at a particular point in a PCR thermal cycling reaction the fluorescent signal

generated at the annealing temperature is compared to the signal generated at the denaturing temperature and a ratio for each cycle is calculated. Thermal cycling treatment is stopped when the ratio of the last cycle exceeds a selected threshold value, T. This provides an end point in the number of cycles performed and confirms the presence of amplified material for further analysis.

Threshold values are dependent on the dye selected, the temperatures used, the concentrations involved and the sensitivity of the instrumentation. All of these are readily determined empirically for a given system by one of ordinary skill. Two suitable algorithms for the calculation of threshold values are given below where reaction conditions require cycling from 72°C to 94°C.

Algorithm I

(1) if FIU72/FIU94 > T then sufficient amplified material detected

where: FIU72 = fluorescent intensity units at 72°C
FIU94 = fluorescent intensity units at 94°C
T = threshold value which varies with target type and fluorometer based on testing with pure cultures and a variety of food matrices calculated as follows:

(2) $T = 1.5\ T_a$

where: T_a = average ratio FIU72/FIU94 below level of detection (i.e., no appreciable amplification) plus 3 standard deviations.

Algorithm II

Alternatively the following algorithm may be used:

(1) if FIU72 - FIU94 > T then sufficient amplified material detected

where: FIU72 = fluorescent intensity units at 72°C
FIU94 = fluorescent intensity units at 94°C
T = threshold value which varies with target type & fluorometer based on testing with pure cultures and a variety of food matrices calculated as follows:

2) $T = 1.5\ Ta'$

where: Ta' = average difference FIU72 - FIU94 below level of detection (i.e., no appreciable amplification) plus 3 standard deviations.

Figure 9 illustrates how the above algorithms may be used to identify the cycle where dsDNA amplified target is first suspected to be observed. Figure 9 is

a plot of the change in fluorescent signal over a forty cycle PCR run on a chicken lysate spiked with 10^5/mL of *S. typhimurium* in the presence of 2 uM YO-PRO-1™. The upper curve shows the ratio calculations (fluorescent intensity units, FIU taken at 72°C divided by the FIU at 94°C. The lower curve shows the differences (D) obtained by subtracting the FIU at 94°C from the FIU at 72°C.

Threshold calculations were determined according to algorithm I using cycles 5-10 where no amplified material had been produced. Over these cycles, the average ratio was 2.566 FIU with a standard deviation of 0.043. Using three standard deviations, 99.7% of the population, T_a was calculated according to algorithm I 2.566 + (3 x 0.043) = 2.695. Thus, a ratio above 2.695 implies that amplification has occurred. However, in order to take the contribution of different matrices into account, the threshold has been empirically adjusted (increased) by the applicants to $T = 1.5\ T_a$ yielding a detection threshold, T, of 4.042. According to Figure 9, the ratio value exceeds the threshold calculation of 4.042 at cycle 24. At this point, additional amplification is not necessary and the sample can be analyzed using the generated waveform as described in later examples.

An alternative calculation can be based on differences (D) in an analogous fashion according to algorithm II. The average for cycles 5 to 10 was 0.080 with a standard deviation of 0.002. Calculating as above (T = 1.5 x (0.080 + 3 x 0.002)), Applicants found T = 0.129. According to Figure 9, at cycle 24, the difference (or delta) exceeds the calculated threshold of 0.129. Again, additional amplification is not necessary after this point and the sample can be analyzed using the generated waveform as later described.

Using this method threshold values for the slope have been determined for three pathogenic bacteria, *E. coli, Salmonella* and *Listeria*. The values were determined by taking the average slope values for all unspiked samples and adding 3X the standard deviation to the mean and calculated for the last minute of the annealing temperature. The threshold slope values for *Salmonella, Listeria monocytogenes* and *E. coli* are 1.15, 0.64 and 0.9 respectively.

EXAMPLE 1

Waveform Generation With 500 Bp DNA, Cycled In The Presence Of Intercalating Agent

Example 1 demonstrates the generation of a specific fluorescence waveform using a 500 bp DNA fragment cycled between 72°C and 94°C in the presence of YO-PRO-1™ dye.

Dye solution was prepared by diluting 1 mM YO-PRO-1™ (Molecular Probes) to a level of 2 uM in lysis buffer (50 mM Tris pH=8.3, 28 mM KCl, 3 mM $MgCl_2$ & 0.1% Triton X-100). 250 ng of 500 bp DNA (Catalogue No. M500 Bioventures) was then added to 50 uL of the dye solution. The resulting

mix was loaded into the TDF system (described above) and cycled as follows: pre-heat 94°C 1 min., 85°C 15 sec, 95°C 45 sec; cycling (94°C 15 sec, 72°C 3 min.) X 5; end 94°C 2 min, return to room temperature. Fluorescence and temperature were recorded throughout the thermal cycling.

Cycling of the 500 bp fragment revealed a waveform which showed a rapid decrease of signal with increasing temperature followed by a slower signal increase as the temperature was reduced (Figure 3). The patterns were reproducible from cycle to cycle and were directly a function of temperature. The temperature affects both intercalation/annealing kinetics as well as dye fluorescence. The data suggests that the temperature-dependent waveform may be used to homogeneously detect the presence of dsDNA.

EXAMPLE 2

Generation Of A Specific Fluorescence Waveform From Amplified Target DNA In Samples Of Food Spiked With *Salmonella*

Example 2 demonstrates that the TDF detection method is capable of distinguishing amplified target DNA in a sample comprising a food matrix and background bacteria and compares favorably with standard agarose electrophoresis and ethidium bromide staining.

The following procedure was used for each food tested. Twenty-five grams of food was weighed out and stomached for 2 min with 225 mL of the BAM specified pre-enrichment media. Following an overnight incubation at 37°C, three 10 mL aliquots were removed and were spiked with *Salmonella* to yield a final concentration of 10^5, 10^4 or 0 CFU/mL. Samples were processed following the standard BAX™ grow back and lysis procedure as described in Example 4.

Briefly, the spiked samples were incubated for 3 h at 37°C. Following the incubation, 5 uL from the 3 h grow back tubes were mixed with 200 uL of lysis reagent (lysis buffer containing 12.5 uL of 10 mg/mL pronase E/mL) containing 2 uM YO-PRO-1™. The lysis tubes were incubated at 37°C for 20 min followed by 10 min at 95°C. For each lysis tube 50 uL was used to hydrate a *Salmonella* specific PCR tablet. The samples were then thermal cycled on the Perkin Elmer 9600 using the following conditions:

94°C	2 min	1 cycle
94°C	15 sec	35 cycles
72°C	3 min	
72°C	7 min	1 cycle
4°C	forever	

Following thermal cycling the samples were subjected to the TDF process as described in Example 1. Some samples were thermal cycled on the TDF system for the complete 40 cycles. The fluorescence data was analyzed and the cycle at which the presence of amplified target was identified. The cycles indicating the presence of amplified target were compared to the absence or presence of the specific product of an agarose gel (Figure 6).

Analysis Of Waveform Generation

Each 94°C to 72°C transition elicited a fluorescence waveform. Such waveforms were extracted from the data files (5/test) and analyzed for Slope, Delta, and Ratio as defined below:

Slope = (C-B)/time Delta = (C-A) Ratio = (C-O/A-O)

where A, B, C are FIU values, C is the peak 72C FIU @tpeak, time is 2 min, B is the FIU @(tpeak-2 min), A is the 94C value @(tpeak-4 min), and O is the fluorometer offset value.

A nominal logistic regression was performed to determine a model that would predict the presence of a band on a gel based on the TDF values of Slope, Delta, and Ratio. Since each food test consisted of 5 waveforms and hence 5 predicted gel results, the final test result was ascertained by what the majority of individual test results indicated.

In order to test samples more efficiently 64 samples were run on a PE9600 for 35 cycles followed by 5 cycles on the TDF system. Based upon agarose gel results, the samples were separated into two categories 1) 54 samples which produced specific bands or no bands and 2) 10 samples which produced nonspecific PCR products. The 54 samples were analyzed for slope, delta, and ratio as described above. Data generated from waveform analysis correlated 100% to the companion data generated from gel electrophoresis. Those samples producing a specific fluorescence waveform (Figure 4) correlated with the presence of amplified target on the gel and those samples non-specific waveform (Figure 5) correlated with the lack of a band on the gel or a smear (Figure 6).

The waveforms generated from the 10 samples that produced nonspecific PCR products were significantly different from the waveforms associated with the 54 samples. A simple algorithm could be devised to distinguish such waveforms.

EXAMPLE 3

Effect Of DNA Fragment Size On Waveform Character

To determine the effect of DNA fragment size on the character of the waveform, DNA fragments of various sizes (1.0-48 kb) were subjected to TDF analysis to determine if there was a correlation between the resultant FIUs

waveform pattern and the size of the DNA fragment. DNA fragments of 1.0, 2.2, 6.4 and 9.6 kb were obtained from the plasmid P104 [derived from pGEM, Promega, MN] containing 3.2 kb insert of DNA. The 1.0 and 2.2 kb fragments are digests of the DNA insert that had been agarose gel purified. The 6.4 kb fragment is the P104 plasmid without the DNA insert and the 9.6 kb fragment is the plasmid containing the insert. In addition, lambda DNA (48 kb obtained from Boehringer Mannheim) was also subjected to the TDF process. The concentration of the DNA fragments was determined by measuring the absorbance at 260 nm and using the extinction coefficient of 50 ug/mL =1.0 optical density units. Approximately 750 ng DNA/50 uL was diluted in lysis buffer containing 2 uM YO-PRO-1™. The DNA solutions were loaded onto the TDF system and cycled as follows:

pre-heat 94°C 1 min, 85°C 15 sec, 95°C 45 sec; cycling (94°C 15 sec, 72°C 3 min) X 5; end 94°C 2 min, return to room temperature.

Fluorescence and temperature were recorded throughout the thermal cycling. Following the thermal cycling both temperature and fluorescent units were plotted and the resultant waveform patterns were compared.

The data showed that the TDF waveform is a function of the size of the DNA fragment. The larger DNA fragments (6.4, 9.6, 48 kb) [Figure 7] generated waveforms that were similar to the waveforms produced by the nonspecific PCR products. The smaller DNA fragments (1.0, 2.2 kb) [Figure 8] generated waveforms similar to those produced by the specific products (750 bases) found in the spiked food samples.

EXAMPLE 4

Differentiation Of Amplified Target And Non-Target DNA

Example 4 illustrates the ability of the present method to differentiate between amplified target dsDNA and non-target DNA comprising a heterogeneous combination of dsDNA, and ssDNA of various sizes such that the mixture appears as a smear of DNA fragments when resolved by electrophoresis.

The smear product was generated from the following protocol:

1. Combine the following for each reaction:
   43 µL water
   5 µL 10X reaction buffer
   1.2 µL Taq dilution buffer
2. Add one *Salmonella* PCR reagent tablet
3. Add 1.0 µL *E. coli* genomic DNA extract (50 ng/µL)
4. Denature at 94°C for 2 min
5. Run 35 cycles of the following program:
   15 sec at 94°C; 10 min at 72°C wherein

Taq Dilution Buffer is:	10 mM Tris @ pH 8.0
1% Tween 20 and	
10X Reaction Buffer is:	100 mM tris pH 8.3
	280 mM KCl
	45 mM $MgCl_2$

Temperatures were cycled between 72°C and 94°C. Table I shows the temperature-dependent fluorescence characteristics of ssDNA, dsDNA and a mixture of PCR-generated products that appears as a smear of DNA fragments. The F @ 1' represents the fluorescence one minute after the temperature was decreased from 94°C to 72°C. All measurements were made with YO-PRO-1™ at a concentration of 2 μM.

Table I: Temperature-Dependent Fluorescence for ss and ds DNA

	Fluorescence(F) @ 1'	Maximum dF/dt	(Maximum dF/dt) (F@1')	(dF/dt) @1'	(dF/dt) (F @1')
ssPhiX174, 15ng/μL	0.088	0.0024	0.027	0.0E+00	0.0E+00
ssPhiX174, 7.5ng/μL	0.038	0.001	0.026	0.0E+00	0.0E+00
1.0X PCR Smear	3.5	0.16	0.046	2.0E-04	5.7E-05
0.5X PCR Smear	1.9	0.13	0.068	1.5E-04	7.9E-05
Salmonella PCR Product Å .75kb	0.4	0.012	0.030	2.3E-03	5.8E-03
dsDNA 1kb, 7.5ng/μL	0.78	0.019	0.024	4.0E-03	5.1E-03

The first two columns of fluorescence data indicate that neither a measurement of absolute fluorescence or maximum rate of change in fluorescence is helpful in fully differentiating homogeneous dsDNA, which is represented by the *Salmonella* PCR product and the 1 kb dsDNA, from the ssDNA and PCR smear product. Normalizing the maximum rate based on the absolute fluorescence at one minute after the temperature decrease to 72°C does not provide any additional discrimination. Measuring the rate of change in fluorescence at one minute after the change to 72°C. (dF/dt @ 1') does provide some differentiation between the homogeneous dsDNA and ssDNA or PCR smear product. This difference can be amplified by normalizing the dF/dt @ 1' based on the absolute fluorescence at one minute after the temperature decrease.

Intermolecular reannealing in homogeneous dsDNA is significantly slower than the intramolecular interactions that cause fluorescence in the ssDNA or PCR

smear product. Because of this effect, reannealing kinetics are still manifest one minute after the temperature has been reduced to the annealing temperature of 72°C. In the ssDNA or PCR smear product the reannealing process is virtually complete after one minute. The net result is that the dF/dt value at one minute is significantly greater in homogeneous dsDNA than in ssDNA or PCR smear product. As such, rate measurements of fluorescence that are made after the ssDNA or PCR smear product reannealing process is nearly complete can be quite useful in discriminating between homogeneous dsDNA and ssDNA or PCR smear products.

EXAMPLE 5

Comparison Of Fluorescent Signal With Standard Sample Preparation Vs. Modified Sample Preparation

Identification Of Target DsDNA In Samples Subjected To Standard BAX™ Sample Preparation Method - turkey

Salmonella typhimurium (DD1084) was spiked into a turkey culture, using the standard BAX™ protocol (Qualicon™) as described below. After sample prepartation the DNA is amplified and detected using the TDF-PCR detection process as described in Example 1.

Food Matrix Preparation

A 25 g sample of aseptically weighed turkey meat was placed into a sterile stomacher bag with 225 mL of sterile lactose broth, and blended constantly for 1 min. The pH of the food mixture was measured with pH paper (5-10 range). Steamed Tergitol Anionic 7 solution (2.25 mL) was then added to the mixture. The mixture was incubated at 35°C for 20 h to permit bacterial growth.

Salmonella Culturing

Using an inoculating loop, one isolated *Salmonella typhimurium* colony (DD 1084) was removed from a BHI agar plate, and inoculated into a culture tube containing 10 mL of BHI broth. This culture was allowed to grow for 22 ± 2 h in a 35°C incubator without agitation. After 22 h, a 1/10 serial dilution was made of the *Salmonella* cultures with 0.1% peptone water to produce the following concentrations: 10^8, 10^9, 10^6, 10^5, 10^4, 10^3 cfu/mL. Three individual 100 µL aliquots of the 10^3 cfu/mL culture sample were then spread onto individual BHI agar plates and incubated at 37°C for 24 h in order to obtain a total plate cell count.

Sample Spiking and Grow Back

Three samples (5 mL each) from the pre-enriched food cultures were placed into 15 mL culture tubes and mixed with 50 µL of 10^7, 10^6, and 0 dilutions of the *Salmonella* DD 1084 culture respectively to achieve the final concentrations of 10^5, 10^4, and 0 cfu/mL of *Salmonella* cell count. A 1 mL sample from each

spiked culture tube was then transferred to a tube containing 9 mL of BHI broth, vortexed and incubated for 3 h at 37°C to allow *Salmonella* growth.

Cell Lysis

A 5 µL aliquot of the spiked culture mixture was placed in a lysis tube containing 195 µL of working YO-PRO-1™/Pronase-E /lysis buffer. (2 µM YO-PRO-1™/200 ng/µL Pronase E/50 mM Tris/3 mM $MgCl_2$/28 mM KCL/0.1% Triton X 100 buffer, pH 8.3) and vortexed. All lysis tubes were placed in a floating rack in a 37°C water bath for 10 min. The lysed sample tubes were then moved to a 95°C water bath for 10 min to inactivate the Pronase E enzymatic activity, then allowed to cool.

PCR Amplification

A 50 µL aliquot of each lysed sample was placed in a PCR tube containing a commercially available BAX™ For Screening/*Salmonella* tablet (Qualicon™), and run in a Perkin-Elmer 9600 Thermal Cycler with the following settings:

Sample vol.:	50 µL
Initiation:	95°C for 2 min
Melting:	95°C for 15 sec, 35 cycles
Annealing and elongation:	72°C for 2 min
Extension:	72°C for 7 min
Holding:	4°C forever

TDF Detection (5 cycles)

A 50 µL aliquot of each lysed sample was placed in a PCR tube containing a commercially available BAX™ For Screening/*Salmonella* tablet (Qualicon™), and run in an in-house prototype instrument using a pair of fluorometers, a thermal cycler, and cooling system. The device was programmed as follows:

- For computer recording:
  - 3 sec/reading
  - 20 point/min
  - Recording time: 0.3 h
  - Dual channel (#12 & 13)
  - Temp. channel #12 only
- For Cycler/Fluorometer:

Sample vol.:	50 µL
Initiation:	94.2°C for 1.3 min, 79.5°C for 30 sec, 94.3°C for 30 sec

Cycles:	95°C for 42 sec, 60°C for 16 sec, 71.6°C for 2.5 min, total 5 cycles
Holding:	94.2°C for 2 min

Interpretation

The waveform was plotted and the slope of each waveform calculated using MicroSoft Excel software. The slope of each waveform is described as: (C-B)/2 min = FIU (Fluorescence Intensity Units)/min., where

C: fluorescent value at the point of transition from 72°C to 94°C

B: fluorescence signal at 2 min prior to the point C

A: fluorescence signal at baseline (94°C).

Any sample with the slope > 0.004 is a positive *Salmonella* sample. Data are contined in Table II.

Identification Of Amplifided Target dsDNA In Samples Subjected To Modified Sample Preparation Method - Chicken

Salmonella typhimurium (DD1084) is spiked into a chicken culture, the sample prepared using the modified sample preparation method that includes addition of RNAse, then amplified and detected using the TDF detection process as described in Example 1.

Food Matrix Preparation, Salmonella Culturing, and Sample Spiking and Grow Back

The food matrix prepearation was acomplihsed as described above.

Cell Lysis

A 500 mL aliquot of the spiked culture mixture was placed in a dilution tube containing 4.5 mL of 0.1% peptone water and vortexed and allowed to rest for 10 min to allow food particles to settle. For each sample, a 30 µL aliquot was removed from the middle of each dilution tube and transferred to a lysis tube containing 163 µL of working YO-PRO-1™/Pronase-E/lysis buffer (3 µM YO-PRO-1/200 ng/µL Pronase E/50 mM Tris/3 mM $MgCl_2$/28 mM KCL/0.1% Triton X 100 buffer, pH 8.3) and vortexed. All lysis tubes were placed in a floating rack in a 37°C water bath for 10 min. The lysed sample tubes were then moved to a 95°C water bath for 10 min to inactivate the Pronase E enzymatic activity, then allowed to cool. For each lysis tube, 7 µL of 350 ng RNAse per 200 µL lysate was added. All the tubes were returned to the 37°C water bath for another 15 minutes incubation.

PCR Amplification

Amplification was accomplished as described above to except for the following settings:

Melting:	95°C for 15 sec, 38 cycles
Annealing and elongation:	72°C for 2 min

TDF Detection (2 cycles)

The procedure was identical as that described above, except that only 2 cycles were run.

Interpretation

The analysis of waveform was identical to that described above for samples treated with the standard sample preparation method. The slope of the waveform is given in Table II indicating a much stronger signal and better discrimination versus the standard preparation procedure.

Identification Of Amplified Target dsDNA In Samples Subjected To Modified Sample Preparation Method - Oregano

Salmonella enteritidis (DD706) is spiked into an oregano culture, the sample prepared using the modified sample preparation method that includes addition of RNAse, then amplified and detected using the TDF detection process as described in Example 1.

Food Matrix Preparation

Oregano was prepared as described above for chicken and turkey except that oregano is used, and trypticase soy broth replaces the lactose broth.

Salmonella Culturing, Sample Spiking and Grow Back, Cell Lysis, PCR Amplification, TDF Detection (2 cycles) and Interpretation

Culturing was done as described above for chicken and turkey except that *Salmonella enteritidis* (DD706) is used.

Table II shows enhanced *Salmonella* detection on the basis of slope of the waveform using the modified method.

Combining the new sample preparation with homogeneous temperature dependent fluorescence -PCR assay permitts the detection a very low level contamination of target bacteria in food samples or the like.

TABLE II

Sample Prep.	Food Type	Target Level (*Salmonella*)	Slope
Std Bax™	G. turkey	0	0.005
Std Bax™	G. turkey	10^4 cfu/mL	0.013
Std Bax™	G. turkey	10^5 cfu/mL	0.021
Modified	Chicken	0	0.001
Modified	Chicken	10^4 cfu/mL	0.021
Modified	Chicken	10^5 cfu/mL	0.028
Modified	Oregano	0	0.000
Modified	Oregano	10^4 cfu/mL	0.017
Modified	Oregano	10^5 cfu/mL	0.039

EXAMPLE 6

The Use Of Elevated Temperature Dependent Fluorescence Reduce The Signal Associated With Primer-Dimer

Example 6 demonstrates that increasing the annealing temperature during the TDF method results in reduced signal associated with primer-dimer formation. The reduction in signal associated with primer-dimers decrease the potential for false positive results in samples that produced large amounts of primer-dimer which did not contain specific target DNA.

Primer-dimer was formed by hydrating 12 *Listeria* spp. tablets with 50 ul of lysis buffer (50 mM Tris pH=8.3, 28 mM KCl, 3 mM $MgCl_2$ & 0.1% Triton X-100. The hydrated tablets were then thermal cycled following the normal *Listeria* spp. thermal cycle protocol:

94°C	2 minutes	1 cycle
94°C	15 seconds	38 cycles
70°C	3 minutes	
72°	7 minutes	1 cycle
4°C	Forever	

Following thermal cycling the reactions were pooled in to a single tube. The production of primer-dimer was confirmed by adding 2 ul of loading dye (15% Ficoll 400, 0.1% SDS, 0.1% xylene cyanol FF and 5 mM EDTA) to 10 ul of pooled product which was then run on a ethidium bromide pre-stained 2% agarose

gel run at 180 volts for 25 minutes. Following electrophoresis, the band was visualized using a UV transilluminator.

A standard curve of a 500 base pair DNA fragment (Catalogue No. M500 Bioventures) was prepared with and without the addition of primer-dimer. The standard curve was prepared by making a 2 ml solution of a 1:45,000 dilution of SybrGreen™ (Molecular Probes, Eugene OR.) in lysis buffer. The SybrGreen™ solution was aliquoted in to 1 x 400 ul aliquot and 5 x 200 ul aliquots. To the 400 ul aliquot was added 80 ul of the 500 base pair DNA. This solution is the equivalent of 1000 ng of DNA per 50 ul. This was then two-fold serially diluted using the 200 ul aliquots. A 50 ul sample from each of the serially diluted tubes was mixed with either 50 ul of diluted SybrGreen™ solution or with 50 ul of primer-dimer containing SybrGreen™. The resulting standard curves had DNA concentrations of 500, 250, 125, 62.5, 31.25, 15.62 and 0 ng of DNA/50 ul with and without primer-dimer.

TDF was performed on a Perkin Elmer 7700 for both standard curves. The conditions used for the TDF were as follows:

Run 1.

94°C	1 minute	1 cycle
94°C	15 seconds	3 cycles
72°C	3 minutes	
25°	Forever	

Run 2.

94°C	1 minute	1 cycle
94°C	15 seconds	3 cycles
75°C	3 minutes	
25°	Forever	

Run 3.

94°C	1 minute	1 cycle
94°C	15 seconds	3 cycles
80°C	3 minutes	
25°	Forever	

Run 4.

94°C	1 minute	1 cycle
94°C	15 seconds	3 cycles
85°C	3 minutes	
25°	Forever	

When TDF was performed at 72°C the sensitivity of detecting the 500 base pair fragment was 15 ng/50 ul in the sample that had no primer-dimer and 62.5 ng/50 ul in the samples containing primer-dimer (Figure 10a, 10b). The decrease in the sensitivity in those samples containing primer-dimer is to due the signal interference associated with primer-dimer. When TDF on the same samples was performed at 75°C the level of sensitivity was 15 ng/50 ul for samples with and without primer-dimer (Figure 10a, 10b). When TDF was performed at or above 80°C the primer-dimer had no effect but the overall sensitivity of detecting the 500 base pair DNA fragment was reduced. Thus, running TDF where the annealing temperature is between the temperatures of 75°-80°C produces the greatest sensitivity for the 500 base fragment and the least amount of interference from primer-dimer.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) ADDRESSEE: E. I. DU PONT DE NEMOURS AND COMPANY
(B) STREET: 1007 MARKET STREET
(C) CITY: WILMINGTON
(D) STATE: DELAWARE
(E) COUNTRY: UNITED STATES OF AMERICA
(F) ZIP: 19898
(G) TELEPHONE: 302-992-4926
(B) TELEFAX: 302-773-0164

(ii) TITLE OF INVENTION: DETECTION OF DOUBLE-STRANDED DNA IN A HOMOGENEOUS SOLUTION

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: FLOPPY DISK
(B) COMPUTER: IBM PC COMPATIBLE
(C) OPERATING SYSTEM: MICROSOFT WINDOWS 95
(D) SOFTWARE: MICROSOFT WORD VERSION 7.0A

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/040,306
(B) FILING DATE: FEBRUARY 14, 1997
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: MAJARIAN, WILLIAM R.
(B) REGISTRATION NO.: 41,173
(C) REFERENCE/DOCKET NUMBER: MD-1069

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TAGCCGGGAC GCTTAATGCG GTTAAC 26

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTTTACCGCT TCCAGTGTGG CCTGAA 26

WHAT IS CLAIMED:

1. A method for distinguishing dsDNA from ssDNA comprising:

(a) generating and recording a fluorescence waveform for a sample mixture by subjecting said mixture to at least one cycle of alternating denaturing and annealing temperatures, said mixture comprising:

(i) a solution suspected of containing dsDNA; and

(ii) an intercalating agent, wherein said intercalating agent generates a detectable signal when bound to a nucleic acid and no signal when unbound;

(b) measuring the rate of change in fluorescence over the annealing portion of the cycle to generate a slope, wherein the slope indicates the presence or absence of dsDNA.

2. The method according to Claim 1 wherein said measuring of the rate of change in fluorescence of step (b) is accomplished during a time when the annealing temperature has a temperature fluctuation of no more than 0.1°C.

3. The method according to Claim 2 wherein said measuring of the rate of change in fluorescence takes place over a time of 15 sec to 3 min.

4. The method according to Claim 1 wherein when the slope of step (b) is greater than about 0.09, the presence of dsDNA is confirmed.

5. The method according to Claim 1 wherein the sample mixture suspected of containing dsDNA is a complex mixture derived from a food or food product.

6. The method according to Claim 1 wherein the dsDNA is target DNA derived from a target bacteria and generated by primer directed amplification.

7. The method according to Claim 1 wherein said intercalating agent is an asymmetrical cyanine dye.

8. The method according to Claim 7 wherein the cyanine dye is a member selected from the group consisting of YO-PRO-1™ and SybrGreen™.

9. The method according to Claim 1 wherein said intercalating agent is at final concentration of about 0.1 uM to about 20 uM.

10. The method according to Claim 1 wherein said intercalating agent has a DNA binding constant of about $1x10^4$ to about $5x10^5$ ($molar^{-1}$).

11. The method according to Claim 1 wherein the denaturing temperature is from about 94°C to about 100°C and wherein the annealing temperature is from about 65°C to about 90°C.

12. The method according to Claim 11 wherein the annealing temperature is about 85°C.

13. The method according to Claim 6 wherein the target DNA is derived from a member selected from the group consisting of bacteria, fungi, viruses, and insect and mammalian cells.

14. A method according to Claim 13 wherein the target nucleic acid is derived from the group consisting of *Salmonella, Listeria* and *E. coli.*

15. A method according to Claim 6 wherein said target DNA is from about 500 bp to about 10,000 bp in length.

16. A method for detecting a double stranded target DNA in a complex sample mixture comprising:

(a) preparing a sample suspected of containing a target bacteria by:

(i) incubating said sample in a pre-enrichment medium to resuscitate target and non target bacteria;

(ii) incubating said resuscitated target and non-target bacteria in a selective medium to enhance the growth of said target bacteria; and

(iii) lysing the enhanced cells of step (iii) in the presence of an effective amount of pronase E and RNAase to release double stranded target DNA resulting in the formation of a complex sample mixture;

(b) amplifying said double stranded target DNA by primer directed amplification in the presence of an effective amount of intercalating agent by subjecting the complex sample mixture of step (iii) to at least one cycle of alternating denaturing and annealing temperatures wherein a fluorescence waveform is generated;

(c) measuring the rate of change in fluorescence of the fluorescence waveform over the annealing portion of the cycle to generate a slope, wherein the slope indicates the presence or absence of double stranded target DNA.

17. The method according to Claim 16 wherein said measuring of the rate of change in fluorescence of step (b) is accomplished during a time when the annealing temperature has a temperature fluctuation of no more than 0.1°C.

18. The method according to Claim 17 wherein said measuring of the rate of change in fluorescence takes place over a time of 15 sec to 3 min.

19. The method according to Claim 16 wherein when the slope of step (b) is greater than about 0.09, the presence of dsDNA target is confirmed.

20. The method according to Claim 16 wherein the intercalating agent is selected from the group consisting of YO-PRO-1™ and SybrGreen™.

21. The method according to Claim 16 wherein said intercalating agent is at final concentration of about 0.1 uM to about 20 uM.

22. The method according to Claim 16 wherein said intercalating agent has a DNA binding constant of about $1x10^4$ to about $5x10^5$ (molar^{-1}).

23. The method according to Claim 16 wherein the denaturing temperature is from about 94°C to about 100°C and wherein the annealing temperature is from about 65°C to about 90°C.

24. The method according to Claim 23 wherein the annealing temperature is about 85°C.

25. The method according to Claim 16 wherein the target nucleic acid is derived from a member selected from the group consisting of bacteria, fungi, viruses, and insect and mammalian cells.

26. The method according to Claim 25 wherein the target nucleic acid is derived from the group consisting of *Salmonella, Listeria* and *E. coli.*

27. The method according to Claim 16 wherein said target DNA is from about 500 bp to about 10,000 bp in length.

Fig. 1

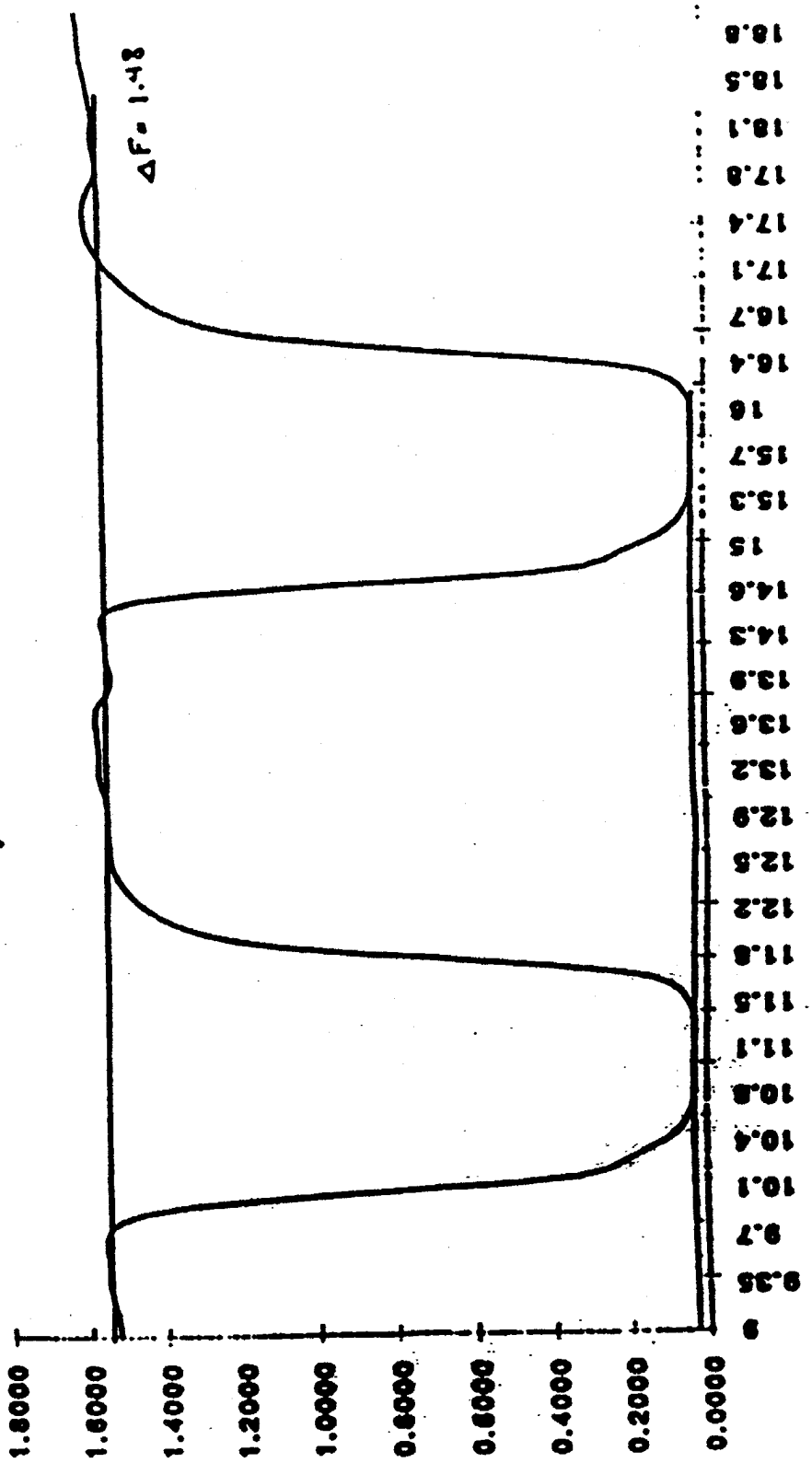
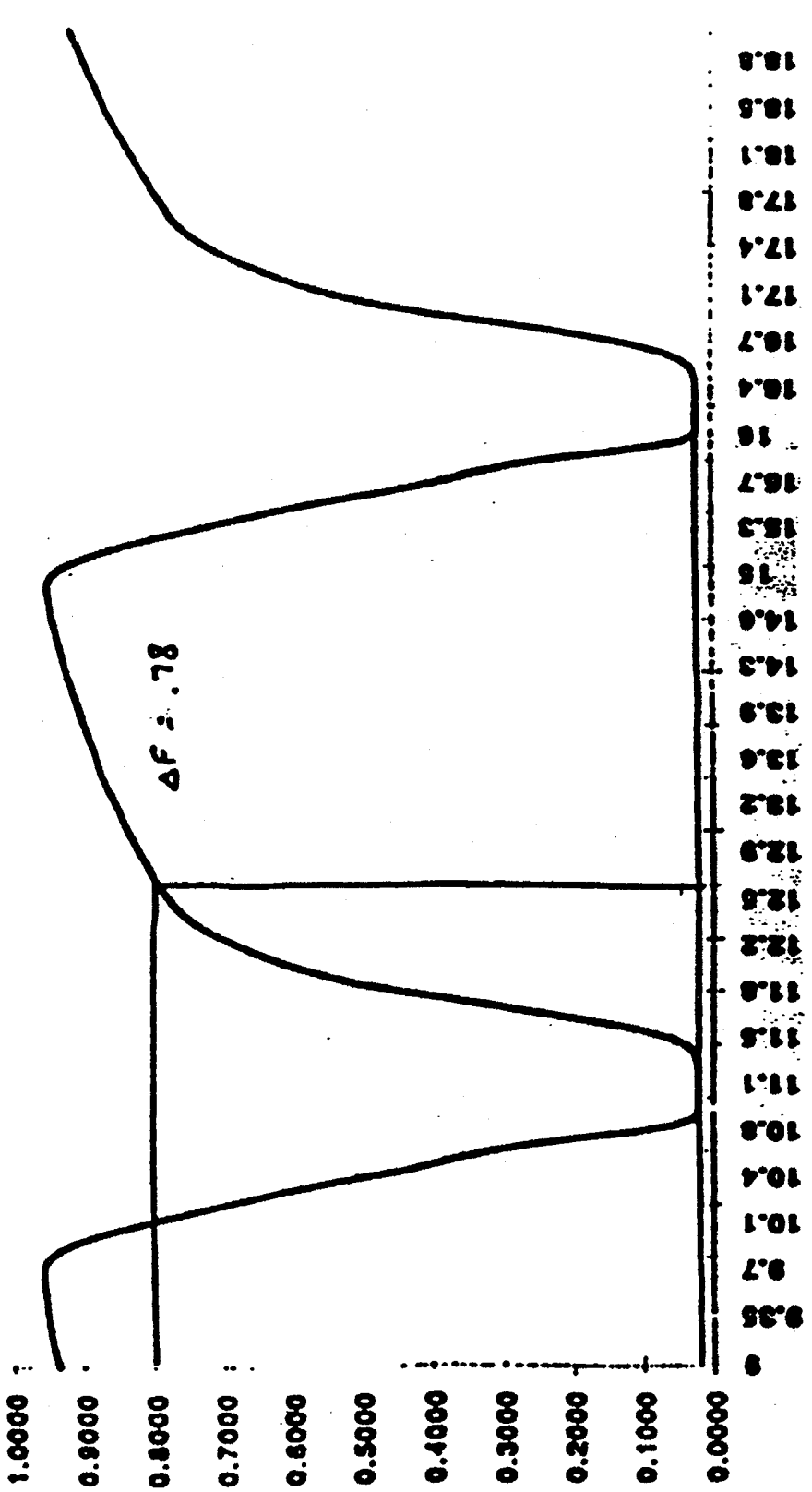

ΔF= 1.48
1.8000
1.6000
1.4000
1.2000
1.0000
0.8000
0.6000
0.4000
0.2000
0.0000
0
9.35
9.7
10.1
10.4
10.8
11.1
11.5
11.8
12.2
12.5
12.9
13.2
13.6
13.9
14.3
14.6
15
15.3
15.7
16
16.4
16.7
17.1
17.4
17.8
18.1
18.5
18.8

Fig. 2


pure dsDNA 1k
375
ΔF = .78
1.0000
0.9000
0.8000
0.7000
0.6000
0.5000
0.4000
0.3000
0.2000
0.1000
0.0000

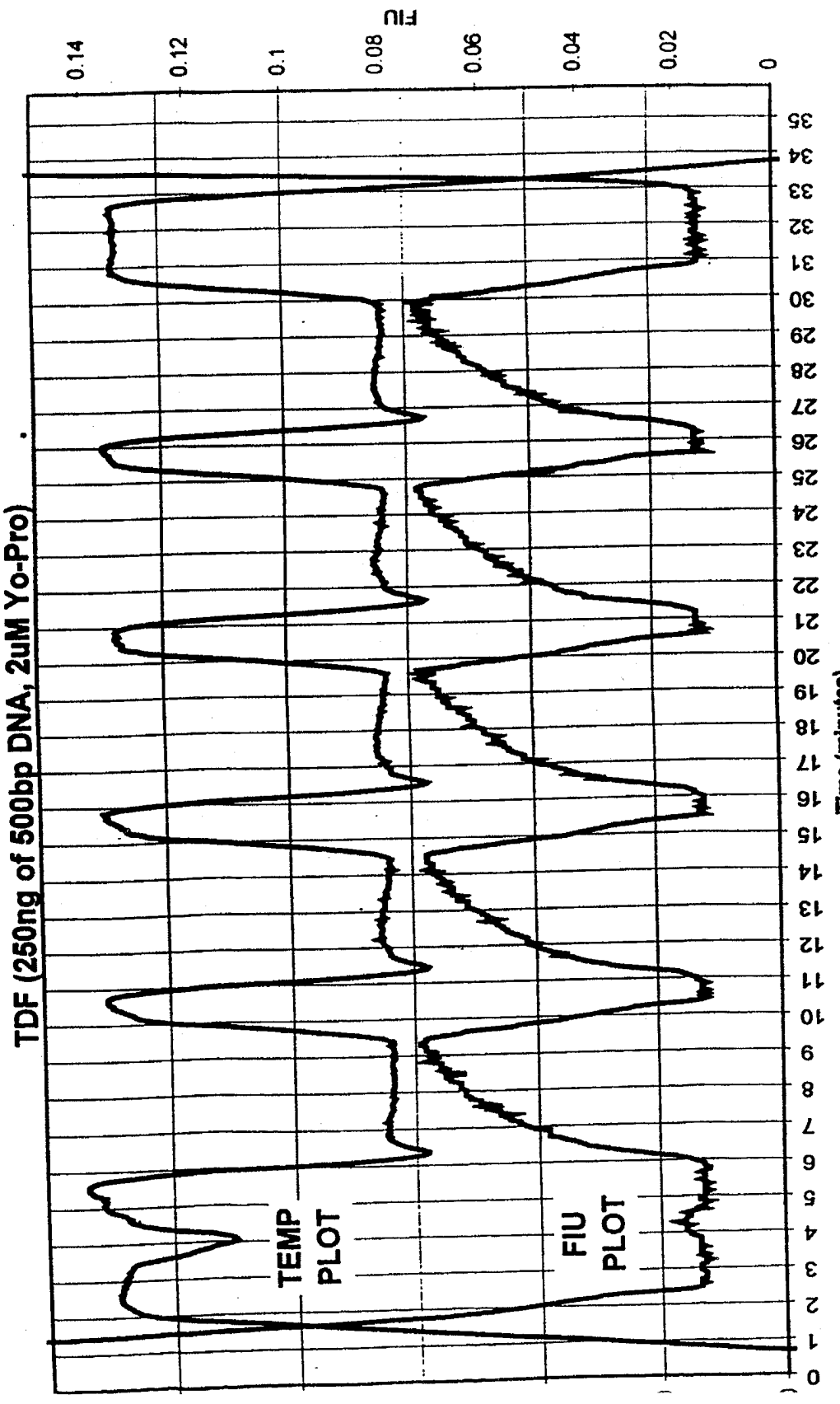

FIU
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
TDF (250ng of 500bp DNA, 2uM Yo-Pro)
TEMP PLOT
FIU PLOT
Time (minutes)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35

Figure 3

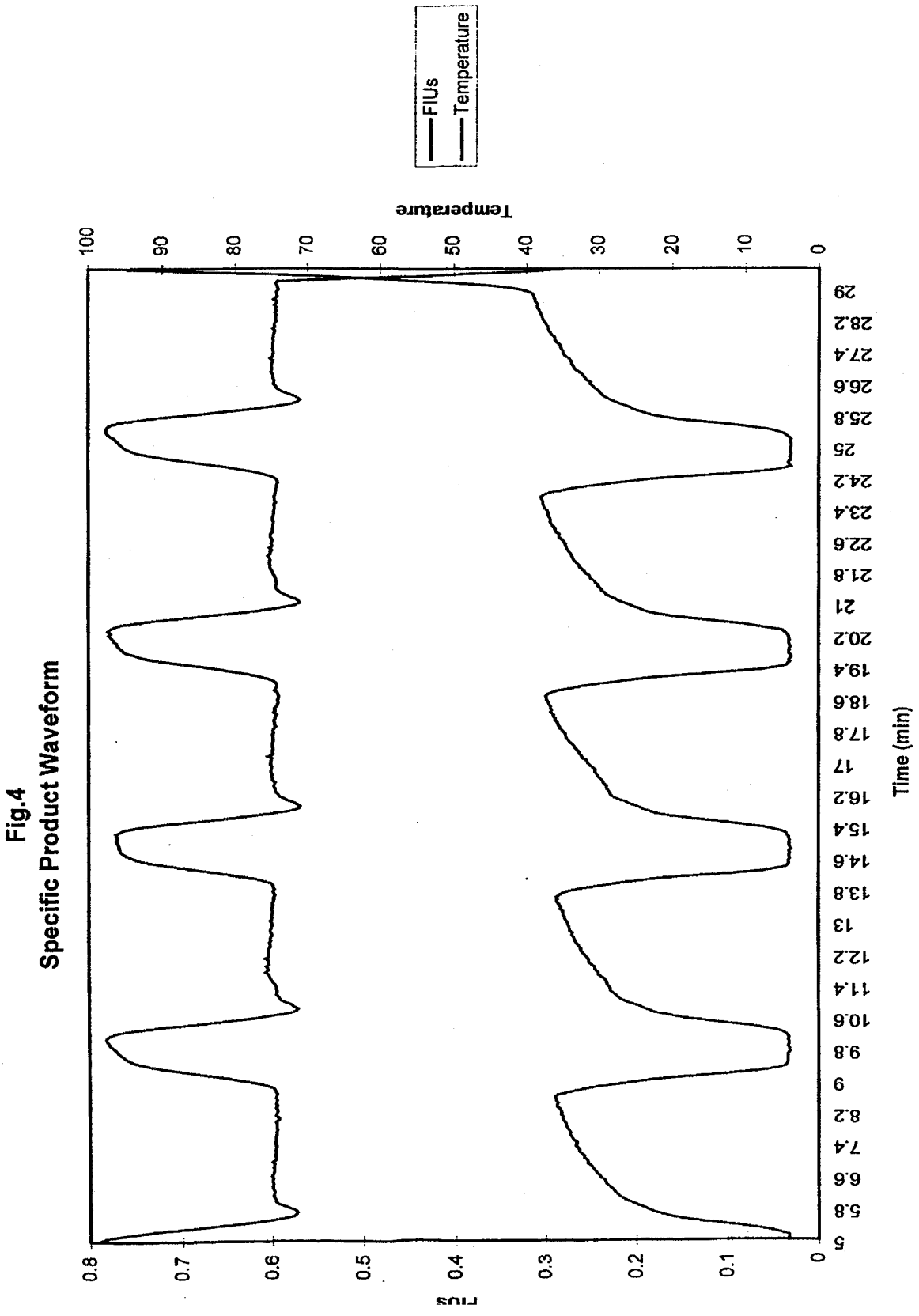


Fig.4
Specific Product Waveform

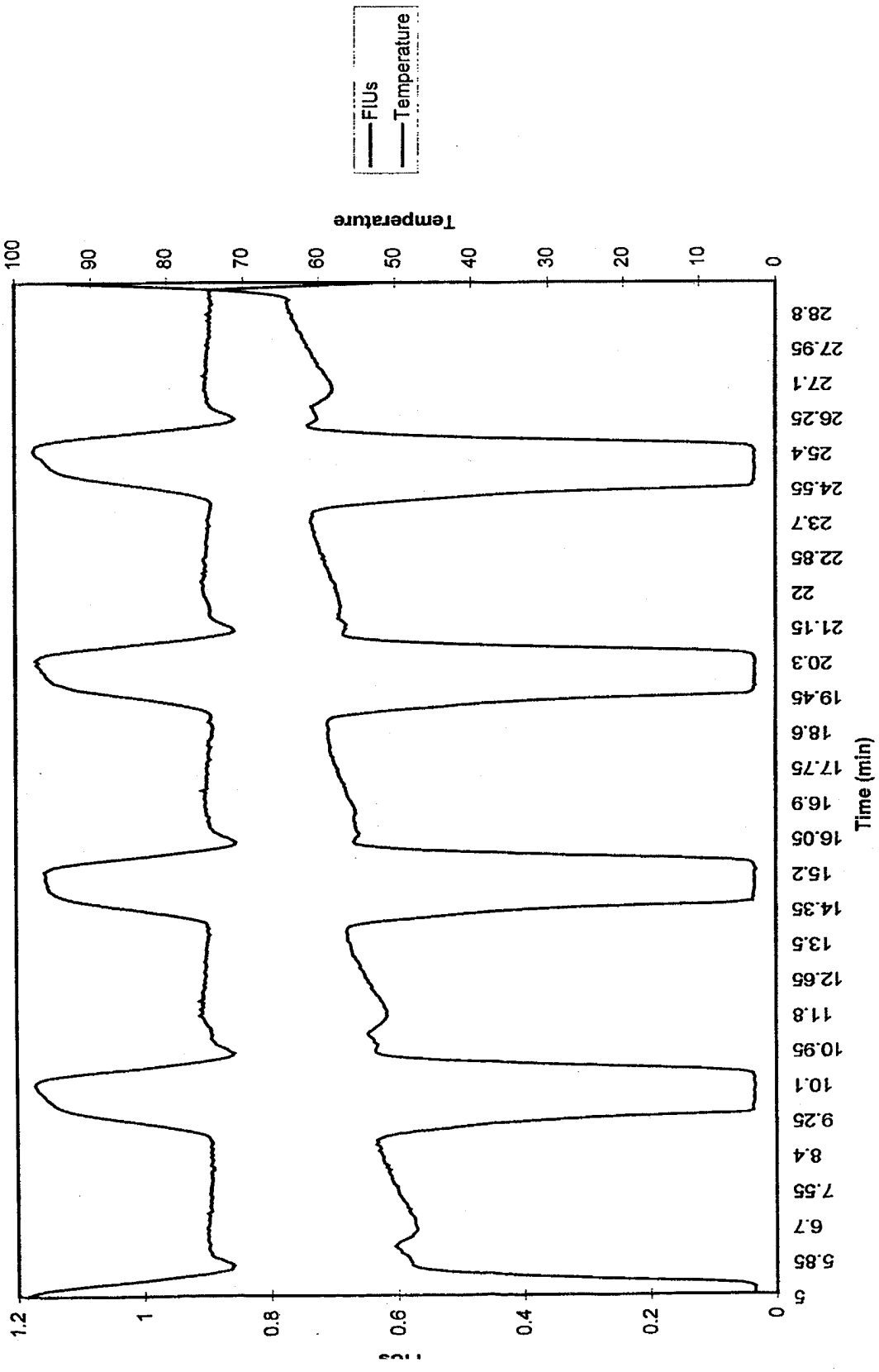


Fig. 5
Non-Specific Product Waveform

Specific and Non-Specific PCR Product

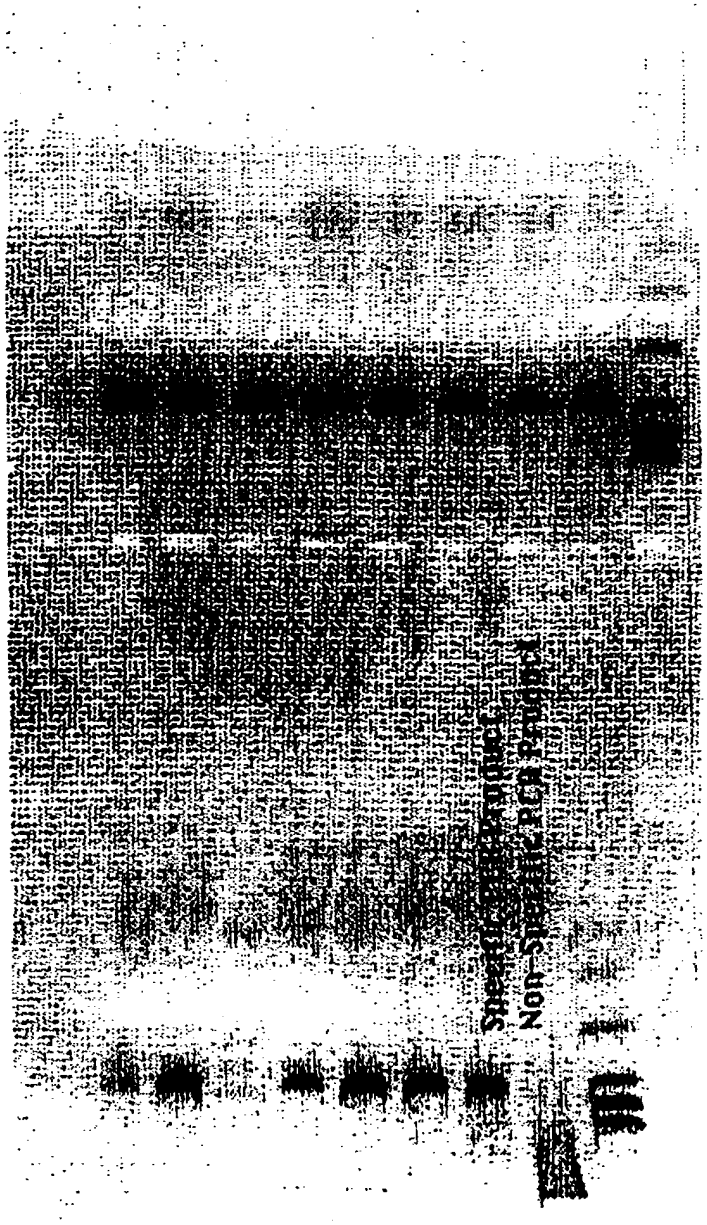


Figure 7a

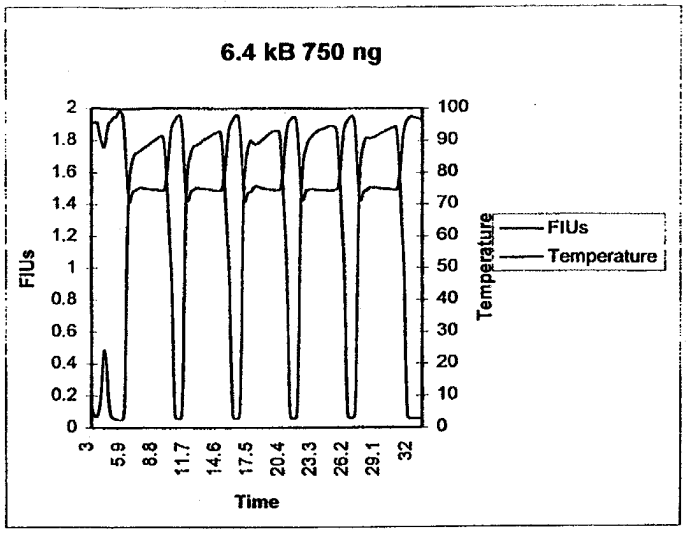


Figure 7b

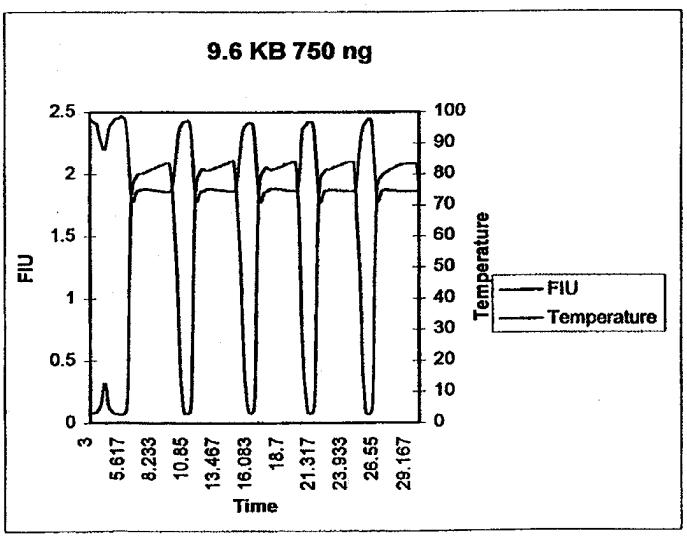
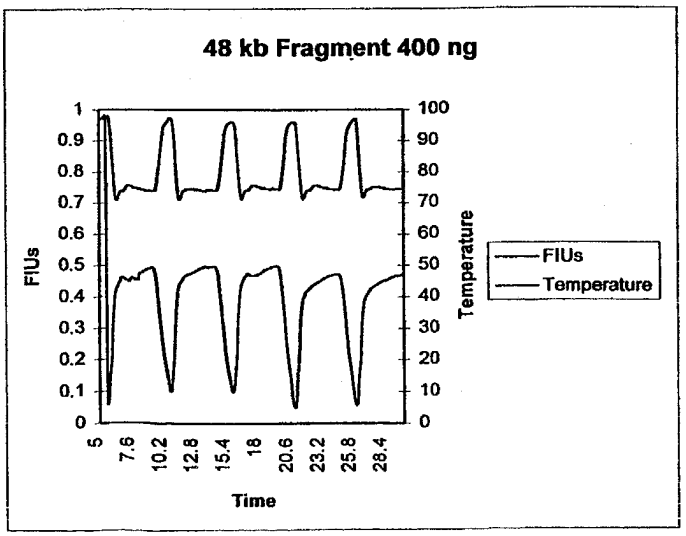


Figure 7c



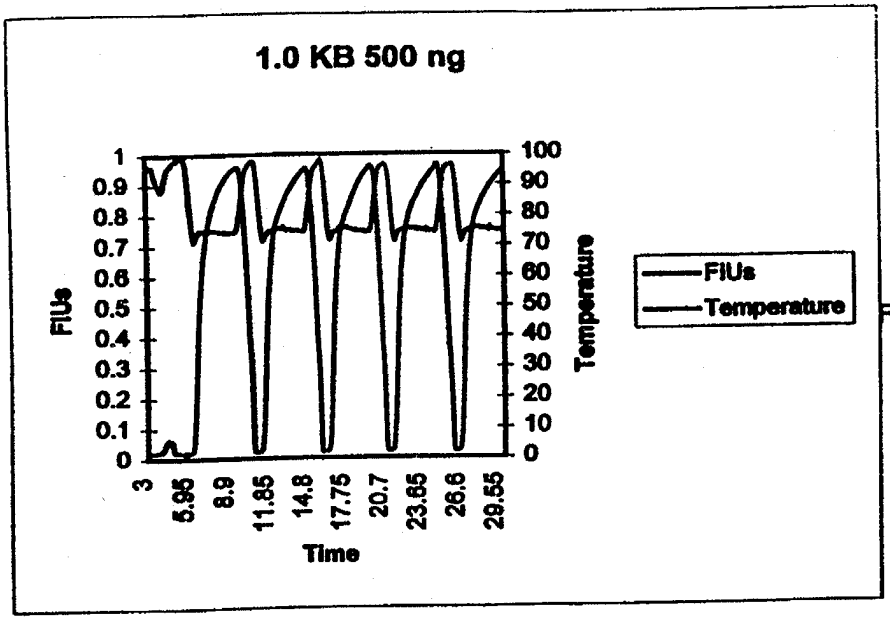


Figure 8a

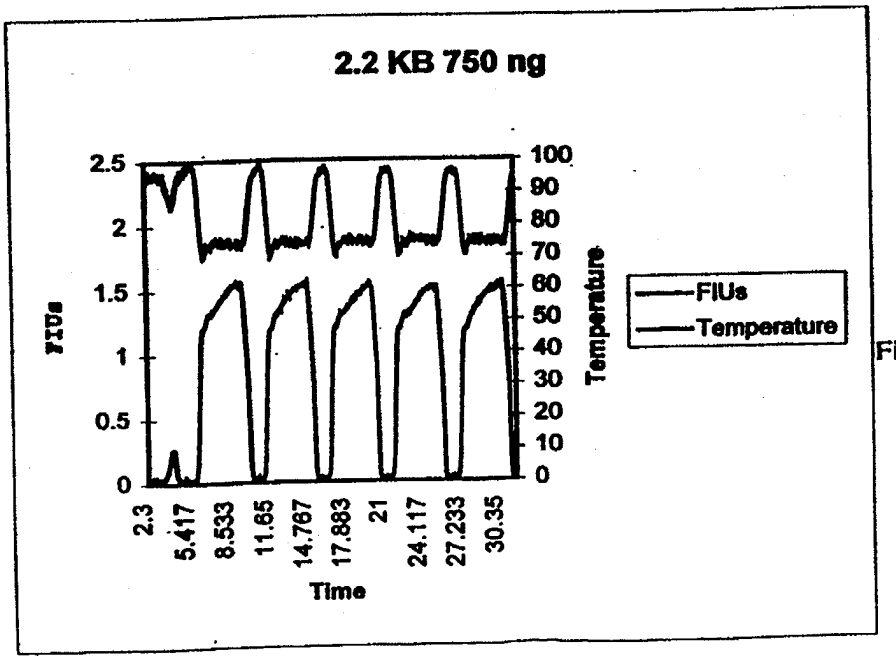


Figure 8b

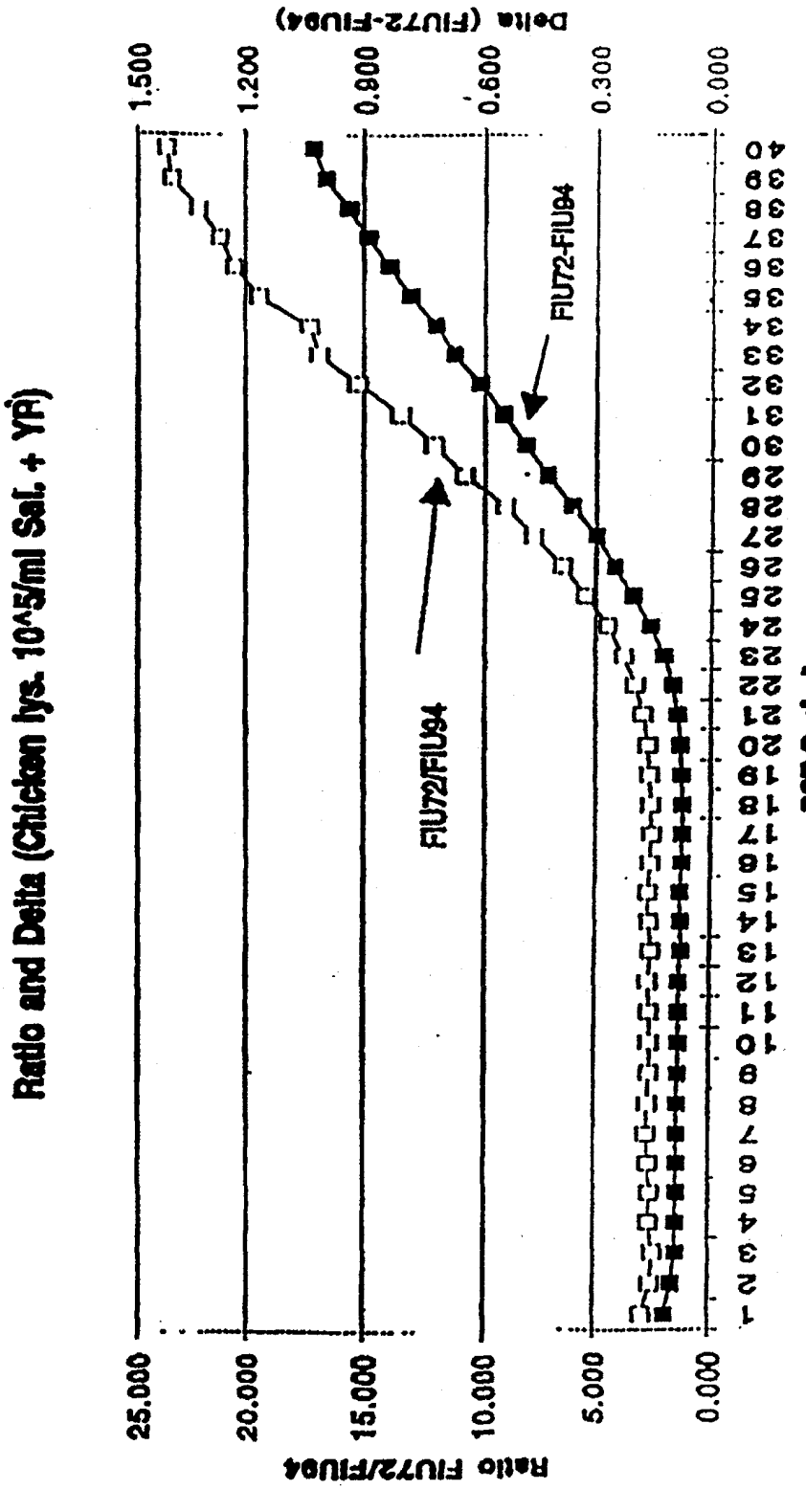

Ratio and Delta (Chicken lys. 10^5/ml Sal. + YR)
Ratio FIU72/FIU94
Delta (FIU72-FIU94)
PCR Cycle #
FIU72/FIU94
FIU72-FIU94
25.000
20.000
15.000
10.000
5.000
0.000
1.500
1.200
0.900
0.600
0.300
0.000

Figure 9

75 Degrees

Fig. 10a

500bp Standard Curve - No Primer Dimer 75 degrees

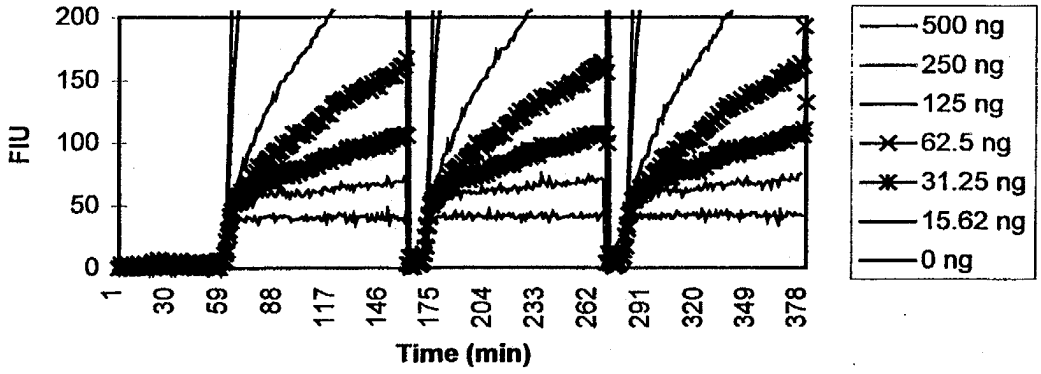
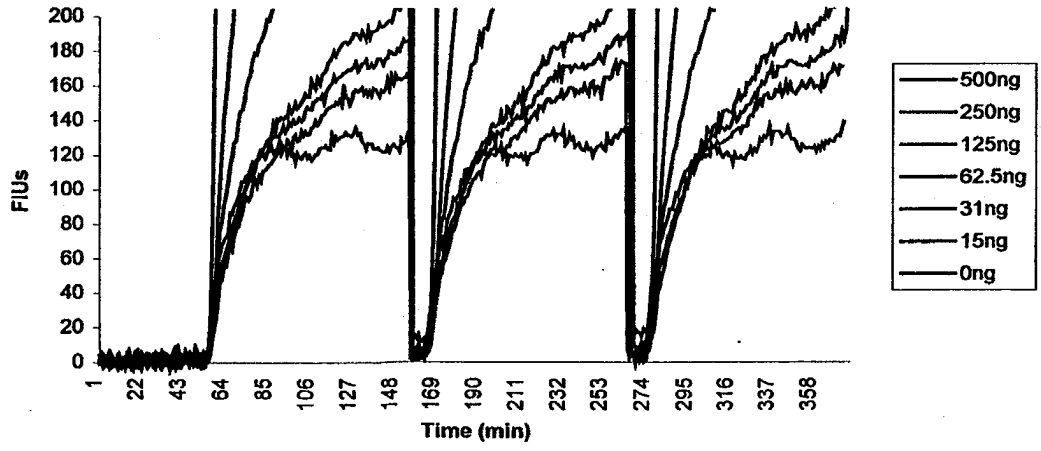


Fig. 10b

75 degrees primer dimer & 500 bp



INTERNATIONAL SEARCH REPORT

Intern. ..onal Application No
PCT/US 98/02750

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HIGUCHI R ET AL: "SIMULTANEOUS AMPLIFICATION AND DETECTION OF SPECIFIC DNA SEQUENCES" BIO/TECHNOLOGY, vol. 10, no. 4, 1 April 1992, pages 413-417, XP000268588 see the whole document	1-27
X	WITTWER C T ET AL: "CONTINUOUS FLUORESCENCE MONITORING OF RAPID CYCLE DNA AMPLIFICATION" BIOTECHNIQUES, vol. 22, no. 1, January 1997, pages 130/131, 134-138, XP000683698 see the whole document	1-27

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 July 1998	09/07/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hagenmaier, S

INTERNATIONAL SEARCH REPORT

Interna. .nal Application No
PCT/US 98/02750

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document. with indication.where appropriate. of the relevant passages	Relevant to claim No.
X	EP 0 640 828 A (HOFFMANN LA ROCHE) 1 March 1995 see the whole document	1-27
X	EP 0 684 316 A (JOHNSON & JOHNSON CLIN DIAG) 29 November 1995 see the whole document	1-27
A	EP 0 643 140 A (CANON KK) 15 March 1995 see the whole document	1-27
A	HIGUCHI R ET AL: "KINETIC PCR ANALYSIS: REAL-TIME MONITORING OF DNA AMPLIFICATION REACTIONS" BIO/TECHNOLOGY, vol. 11, no. 9, September 1993, pages 1026-1030, XP000197685 see the whole document	1-27
A	EP 0 714 986 A (TOSOH CORP) 5 June 1996 see the whole document	1-27
A	WITTWER C T ET AL: "RAPID CYCLE DNA AMPLIFICATION" 1994 , POLYMERASE CHAIN REACTION, PAGE(S) 174 - 181 , MULLIS K B;FERRE F; GIBBS R A XP002044531 see the whole document	1-27
A	EP 0 512 334 A (HOFFMANN LA ROCHE) 11 November 1992 see the whole document	1-27
A	EP 0 487 218 A (TOSOH CORP) 27 May 1992 see the whole document	1-27
P,X	WO 97 46714 A (RASMUSSEN RANDY P ;UNIV UTAH RES FOUND (US); RIRIE KIRK M (US); WI) 11 December 1997 see the whole document	1-27

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internal Application No
PCT/US 98/02750

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0640828 A	01-03-1995	AU 681682 B	04-09-1997
		AU 7141494 A	09-03-1995
		BR 9403338 A	11-04-1995
		CA 2129787 A	28-02-1995
		CN 1107892 A	06-09-1995
		CZ 9402078 A	15-11-1995
		FI 943936 A	28-02-1995
		HU 71622 A	29-01-1996
		JP 7163397 A	27-06-1995
		NO 943166 A	28-02-1995
		PL 304805 A	06-03-1995
		ZA 9406330 A	28-02-1995
EP 0684316 A	29-11-1995	CA 2148140 A	30-10-1995
		CN 1114359 A	03-01-1996
		JP 8089299 A	09-04-1996
		US 5563037 A	08-10-1996
EP 0643140 A	15-03-1995	JP 7163399 A	27-06-1995
		CA 2126391 A	14-03-1995
		US 5670315 A	23-09-1997
		EP 0603783 A	29-06-1994
		JP 9040661 A	10-02-1997
		JP 7174759 A	14-07-1995
		US 5624798 A	29-04-1997
EP 0714986 A	05-06-1996	JP 8211050 A	20-08-1996
EP 0512334 A	11-11-1992	AU 665185 B	21-12-1995
		AU 1513892 A	05-11-1992
		CA 2067909 A	03-11-1992
		JP 5184397 A	27-07-1993
		NZ 242565 A	26-07-1994
EP 0487218 A	27-05-1992	DE 69128520 D	05-02-1998
		JP 5237000 A	17-09-1993
WO 9746714 A	11-12-1997	AU 3154797 A	05-01-1998
		AU 3380097 A	05-01-1998
		AU 3481297 A	05-01-1998

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern... ...nal Application No
PCT/US 98/02750

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9746714 A		WO 9746707 A	11-12-1997
		WO 9746712 A	11-12-1997